Viroid-insect-plant interactions in view of transmission routes

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“Nothing in life is to be feared, it is only to be understood.

Now is the time to understand more, so that we may fear less.”

Marie Skłodowska-Curie

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Viroïden-insect-plant interacties in het licht van transmissierisico's

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Cover illustration:

Left: *Tomato apical stunt viroid* (TASVd) infected tomato plant, Right: The green peach aphid (*Myzus persicae*, Sulzer), flowers of a latently TASVd-infected *Solanum jasminoides* (Paxton), yellowing leaves of a TASVd-infected tomato plant.

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“[…] Twee-, drieduizend jaar geleden bestond er helemaal geen scheikunde en toch vonden de mensen de dingen die ze toen deden ook al vreselijk belangrijk. En nooit stond er iemand op, die zei: “Alles goed en wel, maar ik wou maar dat ik wist hoe ik N-Ethyl-8-hydroxytetrahydrochloropheenhydrochloride moest samenstellen”. Dat kon helemaal niet worden gezegd.[…] Ze wisten feitelijk nog helemaal niet wat materia was en eigenlijk was alles wat erover werd gezegd, onzin. En toch leefden ze. Toch maakten ze geschiedenis. En ze gaven elkaar lauwerkransen. En ze bezongen elkaar in heldendichten."

“Maar ze deden een hoop domme dingen."

“Wij niet dan?”

— Willem Frederik Hermans, Onder professoren

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Contents

List of acronyms and abbreviations

A	
ASBVd	*Avocado sunblotch viroid*
ASSVd	*Apple scar skin viroid*
ANOVA	Analysis of Variance
AP	Acquisition period
B	
BLAST	Basic local alignment tool
C	
ca.	circa
CtRLV	*Carrot red leaf virus*
CBCVd	*Citrus bark-cracking viroid*
CBVd-1	*Coleis blumei viroid 1*
CCCVd	*Coconut cadang-cadang viroid*
CChMVd	*Chrysanthemum chlorotic mottle viroid*
CCR	Central conserved region
cDNA	Complement DNA
CEVd	*Citrus exocortis viroid*
CLSM	Confocal laser scanning microscopy
CLVd	*Columnea latent viroid*
CMV	*Cucumber mosaic cucumovirus*
CP	Coat protein
CRA-W	Walloon Agricultural Research Center
CSVd	*Chrysanthemum stunt viroid*
CVd-V	*Citrus viroid V*
CVd-VI	*Citrus viroid VI*
Ct (= Cq)	Treshold cycle (= quantification cycle)
cv.	Cultivar
D	
DLVd	*Dahlia latent viroid*
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
dNTP	Deoxyribonucleotide triphosphate
ds	Double-stranded
E	
EC	European Commission
EFSA	The European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay

ELVd	*Eggplant latent viroid*
EPPO	European and Mediterranean Plant Protection Organisation
EU	European Union
F	
FASFC	Federal Agency for the Safety of the Food Chain
FISH	Fluorescence in situ hybridization
FW	Forward
H	
HLVd	*Hop latent viroid*
HSVd	*Hop stunt viroid*
I	
ILVO	Institute for Agricultural and Fisheries Research
IRVd-1	*Iresine viroid I*
L	
LME	Linear Mixed Effect (model)
LOD	Limit of Detection
M	
MNase	Micrococcal Nuclease
MPVd	*Mexican papita viroid*
N	
nt	Nucleotide
NGS	Next-gen sequencing
NPPO	National Plant Protection Organization
P	
PaV	*Pariacoto virus*
PBS	Phosphate buffer saline
PCFVd	*Pepper chat fruit viroid*
PCR	Polymerase chain reaction
PepMV	*Pepino mosaic virus*
PLMVd	*Peach latent mosaic viroid*
PLRV	*Potato leafroll virus*
PoLVd	*Portulaca latent viroid*
PPS	Plant Protection Service
PPV	*Plum Pox Virus*
PRA	Pest Risk Assessment
PSTVd	*Potato spindle tuber viroid*
Q	
qPCR	Quantitative polymerase chain reaction

R	
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
R-PAGE	Return-polyacrylamide gel electrophoresis
RTD	Readthrough domain
RT-PCR	Reverse transcription polymerase chain reaction
S	
syn.	Synonym
T	
TASVd	*Tomato apical stunt viroid*
TCDVd	*Tomato chlorotic darf viroid*
TEM	Transmission electron microscopy
TVDV	*Tobacco vein distorting virus*
TPMVd	*Tomato planta macho viroid*
TRSV	*Tobacco ringspot virus*
TYLCV	*Tomato yellow leaf curl virus*

Problem statement and thesis outline

Viroids induce similar symptoms in plants as viruses, but possess totally different structural, functional and evolutionary characteristics that distinguish them very clearly from viruses (Flores *et al*. 2005). Viroids are small, non-protein encoding, single-stranded RNAs that replicate autonomously in plants (Diener 1971). They are classified into two families, the *Avsunviroidae* and the *Pospiviroidae*, consisting of two and five genera, respectively (Flores *et al.* 2001). Viroids cause numerous diseases in economically important plants such as tomato, potato, cucumber, hop as well as in several (sub)tropical and temperate fruits (coconut, avocado, apple, citrus, pear, peach) and ornamentals (chrysanthemums). Symptoms vary depending on viroid (species and variant), host plant (species and cultivar) and environmental conditions (Flores 2001). Infected plants may be symptomless or show dwarfing, flower and fruit deformations, yellowing of the leaves and necrosis (Flores 2001).

Since their discovery in 1971, many questions have been raised concerning viroid epidemiology and more specifically on how viroids are transmitted between plants. In the past decade, several members belonging to the genus *Pospiviroid* have been found in many symptomless solanaceous ornamentals all over Europe. The concern was that from this pool of latently infected hosts, viroids could spread to other solanaceous plants, such as tomato, pepper and potato, in which disease symptoms are formed. To date, the importance of mechanical transmission, transmission through infected seed, pollen and cuttings has been clearly demonstrated by numerous studies. Other transmission pathways for viroids could involve naturally occurring hosts (like weeds), insect vectors, and even associations with plant viruses.

The main goal of this PhD study is to shed light on these other pathways of viroid transmission and gain more knowledge on pospiviroid epidemiology in Belgium. The studies reported in this thesis were conducted with members of the Genus *Pospiviroid* (hereafter referred to as “pospiviroids”) of the family *Pospiviroidae*, which are most prevalently found in Europe. Since pospiviroids are known to affect solanaceous plants that are cultivated for both food consumption (e.g. tomato) and ornamental purposes e.g. *Solanum jasminoides* (Paxton) (EFSA Panel on Plant Health, 2011), the scope of this PhD study spans both production sectors.

The first Chapter of this PhD thesis provides a general introduction to (pospi)viroid epidemiology, with a special focus on insect transmission (**Chapter 1**). **Chapters 2** to **6** report the research conducted from 2012 until 2016 within the framework of this PhD (Figure I).

Each of these chapters centers around a different epidemiological issue: from the role of weed reservoirs in pospiviroid epidemiology (Chapter 2) to interactions of pospiviroids with insects (Chapters 3-5) and the potential encapsidation of pospiviroids by viruses (Chapter 6). Hence, these Chapters span three different types of interactions of the pathogen-vector-host triangle: Pospiviroid-Host, Pospiviroid-Insect and Pospiviroid-Luteovirus interactions (Figure I).

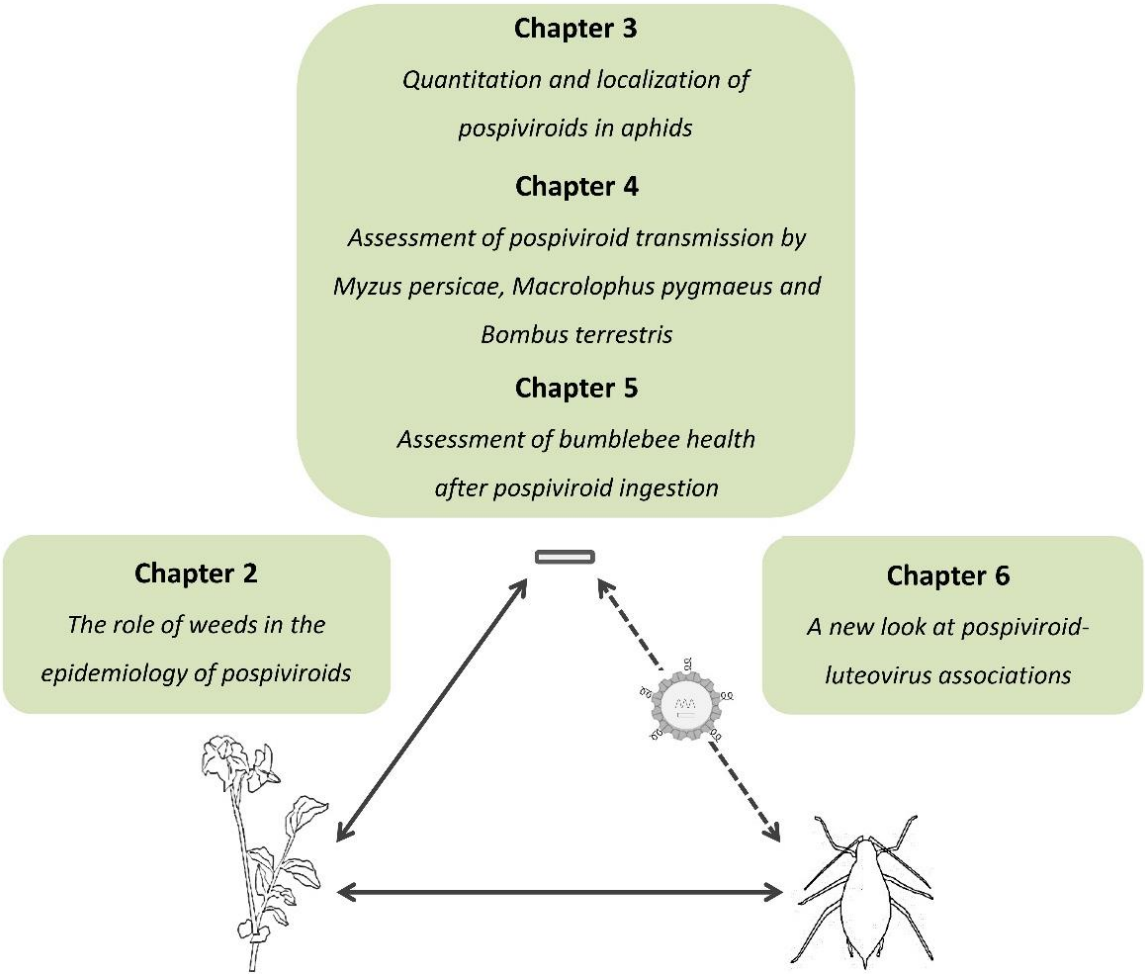


Figure I: Schematic overview of the six research Chapters of this PhD.

The title and rationale behind each of the research Chapters are listed below:

- **The role of weeds in the epidemiology of pospiviroids (Chapter 2)**: Can naturally occurring weed species in a greenhouse environment act as reservoirs for pospiviroids? This question was addressed by organizing a survey of weed species in commercial greenhouses, a contact experiment in a greenhouse and an inoculation experiment.

In this Chapter, the results gained from our experiments were discussed together with results from other inoculation studies in order to get a comprehensive view on the subject of weed reservoirs as potential pospiviroid hosts.

- **Quantitation and localization of pospiviroids in aphids (Chapter 3):** Which methodologies can be used to detect pospiviroids in insects feeding on pospiviroid-infected plants? Where can pospiviroids be localized within the insect's body? In this Chapter, two different techniques were used to localize and quantify viroids : fluorescent *in situ* hybridization (FISH) in conjunction with confocal microscopy and qPCR, respectively. The green peach aphid *Myzus persicae* (Sulzer) was selected as a typical insect vector model for its widespread presence in susceptible crops and its well-established role as plant virus vector. The technical skills and knowledge obtained in this Chapter were essential for further transmission tests with insects (Chapter 4).

- **Assessment of pospiviroid transmission by *Myzus persicae*, *Macrolophus pygmaeus* and *Bombus terrestris* (Chapter 4)**: Can typical greenhouse insects play a role in the dissemination of pospiviroids? In a Pest Risk Assessment (PRA) for pospiviroids in 2011, EFSA stated that there was a high uncertainty concerning insect transmission of pospiviroids due to a limited number of data/studies on this topic and a shortage of different virus/viroid/host combinations tested. Hence, in this Chapter, it was our goal to further investigate the topic of pospiviroid transmission by insects. In contrast to previously conducted insect transmission studies (Antignus *et al.* 2007, Matsuura *et al*. 2010, Nielsen *et al*. 2012) both intra-and interspecies transmission were investigated, different pospiviroid species were used (*Potato spindle tuber viroid* - PSTVd, *Tomato apical stunt viroid* - TASVd, *Tomato chlorotic dwarf viroid* – TCDVd and *Pepper chat fruit viroid* - PCFVd) and the biological control agent *Macrolophus pygmaeus* (Rambur) was tested as a potential vectoring species. Apart from *M. pygmaeus*, two other insect species, belonging to two different functional groups, were used: *M. persicae*, representing the pest insects and *Bombus terrestris* (L.), representing the pollinators. These transmission experiments were necessary in order to evaluate the risk that pospiviroids would spread from infected solanaceous ornamentals to vulnerable crops like tomato using insects as a vehicle.

- **Assessment of bumblebee health after pospiviroid ingestion (Chapter 5):** Are viroids detected in bumblebee progeny after prolonged feeding on pospiviroid-infected pollen? Does feeding on infected pollen lead to any deleterious effects on bumblebee colony development? A recent study by Li *et al.* (2014) showed that the plant virus *Tobacco ringspot virus* (TRSV) infects bees after exposure to virus-contaminated pollen, illustrating that also plant viruses may contribute to pollinator declines. Because of the imperative role that commercial pollinator hives fulfill in greenhouses, it was important to assess whether pospiviroids can sustain themselves in bumblebee bodies after they have been ingested during feeding. In addition, it was investigated whether bumblebees are experiencing any health effects as a result of feeding on pospiviroid (TASVd) - infected pollen. To test this, two experiments were organized in which infected pospiviroid pollen was administered to bumblebee microcolonies which were monitored during a period of 50 days.
- **A new look at pospiviroid-luteovirus associations (Chapter 6):** Are other pospiviroids than PSTVd also transmitted through "transencapsidation" in a virus particle and aphid transmission? In addition to the impact study of direct transmission of pospiviroids via aphids in Chapter 3, it was important to investigate the potential role of an "indirect" transmission route, i.e. transencapsidation, a phenomenon that was described for *Potato spindle tuber viroid* (PSTVd) and *Potato leafroll virus* (PLRV) more than 15 years ago (Querci *et al.* 1997, Salazar *et al.* 1995, Syller & Marczewski 2001). In this Chapter it was our goal to investigate this phenomenon for two other pospiviroids than PSTVd, namely TASVd and TCDVd, which share similar epidemiological (host range, symptoms) and sequence characteristics (large sequence homology, similar secondary structure) with PSTVd. In order to test whether transencapsidation also occurred for these pospiviroid species the enzyme-based methodology of Querci *et al.* (1997) was evaluated and new transmission tests were performed.

In the General Discussion of this thesis (**Chapter 7**) the major research findings of each Chapter and future perspectives are discussed. Based on the knowledge obtained in the research Chapters, risks associated to pospiviroid presence in Belgium are evaluated. Additionally, an outlook on the future regulatory status of pospiviroids in the EU and an overview of phytosanitary options are provided.

Chapter 1:

General introduction

Modified from: Van Bogaert, N., Smagghe, G., De Jonghe, K. (2014). Viroid–insect–plant interactions. In R. K. Gaur, T. Hohn and P. Sharma (editors), *Plant Virus-Host Interaction: Molecular Approaches and Viral Evolution*. Academic Press.

1.1 WHAT ARE VIROIDS?

In the beginning of the 1970s evidence from several independent laboratories indicated that low-molecular weight RNA's, and not viruses, were responsible for diseases observed in potato ("potato spindle tuber disease"; Diener 1971) and in citrus ("citrus exocortis disease"; Semancik & Weathers 1972). Because of the unconventional nature of these entities, new names were proposed and eventually the term "viroid" was accepted. After more than 40 years of research, we now know that viroids are non-protein encoding and highly-structured, single-stranded RNA molecules that cause disease of considerable economic importance (Diener 1971, Diener 2003). Viroid-induced symptoms depend largely on the host plant and the viroid in question, but are usually characterized by diminished growth, stunting, leaf epinasty, necrosis and flower and fruit deformations (Owens & Hammond 2009, Figure 1.1).

Figure 1.1: Typical symptoms of pospiviroids. A) A non-infected tomato fruit vs. a *Tomato apical stunt viroid* (TASVd) infected tomato fruit (*Solanum lycopersicum*, L. Cv. Marmande), B) Curling and chlorosis of the leaves of a TASVd-infected tomato plant (Cv. Marmande), C) Dwarfing and premature flowering of infected Chrysanthemums (Photo: CRA-W, Belgium), D) Latent TASVd-infection in the ornamental plant *Solanum jasminoides* (Paxton).

All presently known viroids vary in length from 246 to 401 nucleotides and display extensive internal base pairing. A tenfold smaller than the genome of the smallest RNA viruses, they are currently considered as the tiniest plant pathogens on Earth (Flores *et al*. 2005). The absence of a protein coat distinguishes viroids from viruses. Furthermore, viroids do not encode for specific proteins, which is why they are relying almost entirely on host factors in order to complete their infectious cycle within the plant (Flores *et al*. 2005).

Certain small satellite RNAs, which are dependent of a helper virus for their replication and encapsidation, exhibit many similar structural features as viroids (Diener 1989). Unlike viroids, however, their replication requires the presence of a specific helper virus (Rubino *et al.* 2003). The RNAs of *Human hepatitis delta virus* (HDV), responsible for Hepatitis D in humans, display common structural (e.g. rod-like secondary structure) and functional characteristics (e.g. ribozymes) with some viroids (Flores *et al.* 2012). In contrast to viroids, the HDV genome codes for a protein (Di Serio *et al.* 2014). These shared properties between viroids and small RNAs support the hypothesis that viroids may have an ancient evolutionary origin independent of viruses, going back to the RNA world postulated to have preceded the present world on Earth based on DNA and proteins (Flores *et al.* 2014, Palukaitis 2014). With the discovery that certain RNAs possess catalytic properties, earlier suggestions that RNA preceded DNA as the carrier of genetic information have gained considerable credence (Diener 1989). The most compelling indication for this is that RNA is the only known macromolecule that can function both as genotype and phenotype, thus permitting evolution to occur at the molecular level in the absence of DNA or functional proteins (Diener 1989). Despite containing only four different chemical subunits, RNA molecules can fold into a variety of complex tertiary structures, analogous to structured proteins and catalyse various chemical reactions, such as site-specific self-cleavage, nucleotide synthesis, RNA polymerization, and peptide bond formation (see reviews of Joyce *et al.* 2002 and Doudna & Cech 2002). However, insight into the origin and operation of the “RNA world” is still largely inferential, i.e. based on the known chemical and biochemical properties of RNA, and future studies will need to sharpen the picture of ancestral RNA-based life through combined efforts in prebiotic chemistry, *in vitro* evolution, biochemical analysis and molecular phylogenetics (Joyce *et al.* 2002).

1.2 CLASSIFICATION

To distinguish viroids from viruses, the suffix “d” is added to the abbreviated viroid name: e.g. *Potato spindle tuber viroid* (PSTVd). Based on biochemical and structural characteristics, viroids are taxonomically divided into two families: *Avsunviroidae* and *Pospiviroidae* (Hadidi *et al.* 2003). Rod-like structures are typical for the *Pospiviroidae*, whereas more branched structures are typical for *Avsunviroidae* (Codoner *et al.* 2006). Another key difference between the two families is the location of replication: *Avsunviroidae* replicate in the chloroplast, while *Pospiviroidae* reproduce within the nucleus (Flores *et al.* 2005). The first discovered viroid, and the type-species of the pospiviroids, is the *Potato spindle tuber viroid* (PSTVd; Diener 1971, Figure 1.2).

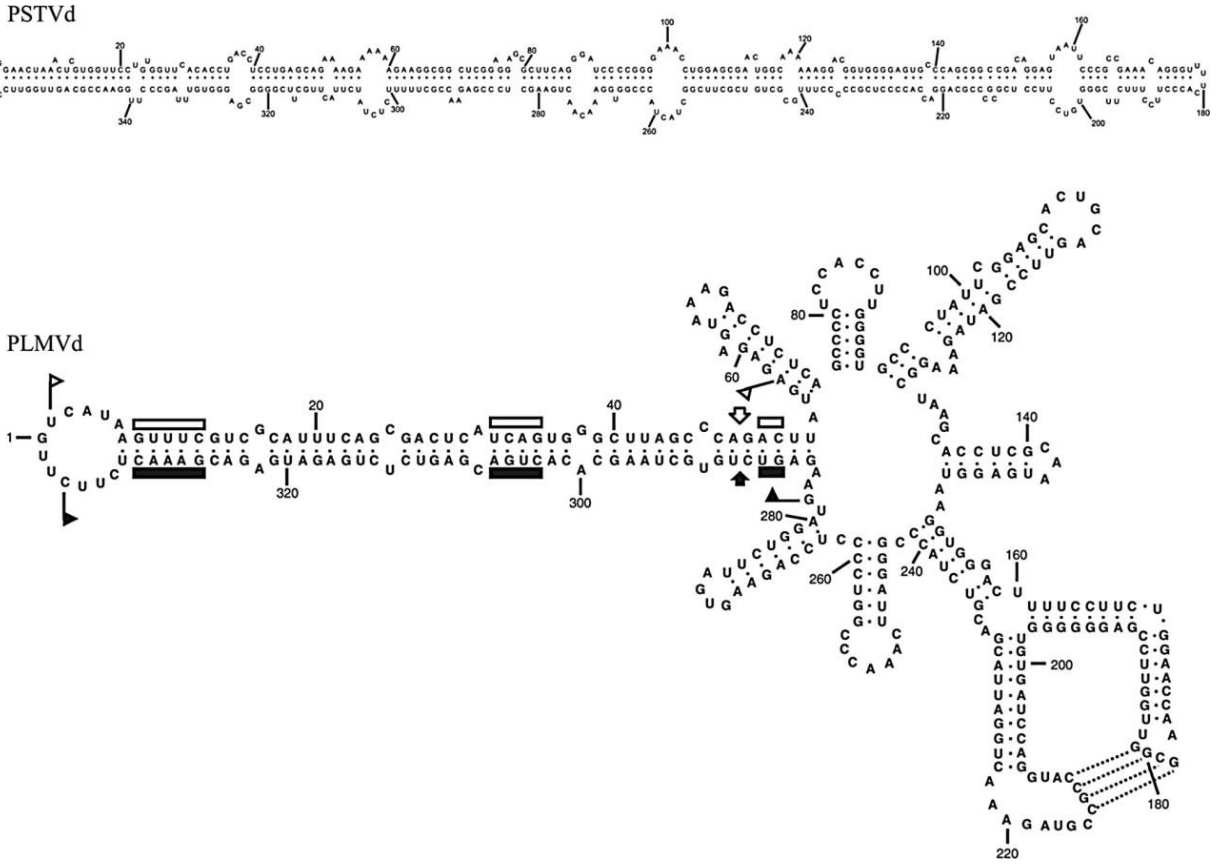


Figure 1.2: Primary and secondary structures of the pospiviroid PSTVd (above) and the avsunviroids *Peach latent mosaic viroid* (PLMVd, below) (Di Serio *et al.* 2014).

To be considered a viroid, two criteria – an arbitrary level of less than 90% sequence identity over the entire genomes and distinct biological properties, particularly host range and symptoms – should be met (Owens *et al.* 2012a).

Table 1.1 shows the most recent taxonomy of viroids (Di Serio *et al.* 2014).

Table 1.1: Current taxonomy of viroids. Modified from: Di Serio *et al.* 2014.

Family	Genus	Species
Pospiviroidae	*Pospiviroid*	*Chrysanthemum stunt viroid*
		Citrus exocortis viroid
		Columnea latent viroid
		Iresine viroid 1
		Mexican papita viroid
		Pepper chat fruit viroid
		Potato spindle tuber viroid
		Tomato apical stunt viroid
		Tomato chlorotic dwarf viroid
		Tomato planta macho viroid
	Hostuviroid	*Hop stunt viroid*
	Cocadviroid	*Citrus bark cracking viroid*
		Coconut cadang-cadang viroid
		Coconut tinangaja viroid
		Hop latent viroid
	Apscaviroid	*Apple dimple fruit viroid*
		Apple scar skin viroid
		Australian grapevine viroid
		Citrus bent leaf viroid
		Citrus dwarfing viroid
		Citrus viroid V
		Citrus viroid VI
		Grapevine yellow speckle viroid 1
		Grapevine yellow speckle viroid 2
		Pear blister canker viroid
	Coleviroid	*Coleus blumei viroid 1*
		Coleus blumei viroid 2
		Coleus blumei viroid 3
Avsunviroidae	*Avsunviroid*	*Avocado sunblotch viroid*
	Pelamoviroid	*Chrysanthemum chlorotic mottle*
		Peach latent mosaic viroid
	Elaviroid	*Eggplant latent viroid*

Most of the 32 currently known viroid species belong to the family *Pospiviroidae*, which comprises 5 genera, among which the genus *Pospiviroid* (Table 1.1). Members of this family differ from members belonging to the family of the *Avsunviroidae*, which comprises three genera, in a number of molecular, biochemical and biological characteristics (Table 1.2). Other differences between members of the *Pospiviroidae* and *Avsunviroidae* are the nuclear RNA polymerases II in the family of the *Pospiviroidae* and plastid- associated RNA polymerases (family *Avsunviroidae*) to accept RNA templates (Flores *et al*. 2005). Additionally, different conserved RNA motifs are involved in the replication of pospi-and avsunviroids: for members belonging to the family of the *Pospiviroidae*, the sequence and structural conservation of the CCR-region is essential for processing of the viroid, while members of the *Avsunviroidae* rely on the hammerhead structures formed by certain small conserved motifs (Flores *et al.* 2000). These hammerhead motifs (named after the similarity of their secondary structure with the hammerhead shark) are RNA sequence motifs that can catalyze self-cleavage at specific sites (Flores *et al.* 2000). Furthermore, members of the *Pospiviroidae* have wide host ranges among the angiosperms, but members belonging to the *Avsunviroidae* have narrow host ranges: they infect only the plants wherein they were discoved and related species (Singh *et al.* 2003, Di Serio *et al.* 2014, Molina-Serrano *et al.* 2007).

Table 1.2: Main differences between members of the family *Pospiviroidae* and *Avsunviroidae*.

Feature	***Pospiviroidae***	***Avsunviroidae***
Secondary structure	Rod	Branched
Location of replication	Nucleus	Chloroplast
Self-cleavage using hammerhead ribozymes	No	Yes
Host-range	Broad	Small

The type-species of the family of the *Pospiviroidae* PSTVd, for example, infects numerous species within the families *Solanaceae*, *Asteraceae, Boraginaceae*, *Campanulaceae*, *Caryophyllaceae*, *Convolvulaceae*, *Dipsaceae*, *Sapindaceae*, *Scrophulariaceae* and *Valerianaceae* (Singh *et al.* 2003). Recently, two new latently present pospiviroids have been discovered in the Netherlands: *Dahlia latent viroid* (DLVd) and *Portulaca latent viroid* (PoLVd) (Verhoeven *et al.* 2013, Verhoeven *et al.* 2015). DLVd is expected to be incorporated in the genus *Hostuviroid* (Verhoeven *et al.* 2013). PoLVd, shows the highest sequence similarity (ca. 80%) with *Iresine viroid 1* (IrVd-1), however, biological differences between the two viroids have not been shown thus far (Verhoeven *et al.* 2015).

1.3 REPLICATION AND PATHOGENESIS

Viroid replication occurs through an RNA-based rolling-circle mechanism that starts with the transcription of the incoming circular positive-sense template to produce oligomeric intermediates that act as templates for a second RNA-RNA transcription (Daròs *et al.* 2006; Figure 1.3). Cleavage and ligation of pospiviroid replication intermediates are catalyzed by host enzymes, whereas hammerhead ribozymes are responsible for self-cleavage of members of the avsunviroids (Figure 1.3). Two host-encoded enzymes, namely the RNA polymerase II and a nuclear-encoded chloroplastic RNA polymerase, are redirected to accept RNA instead of DNA templates (Navarro *et al.* 2012).

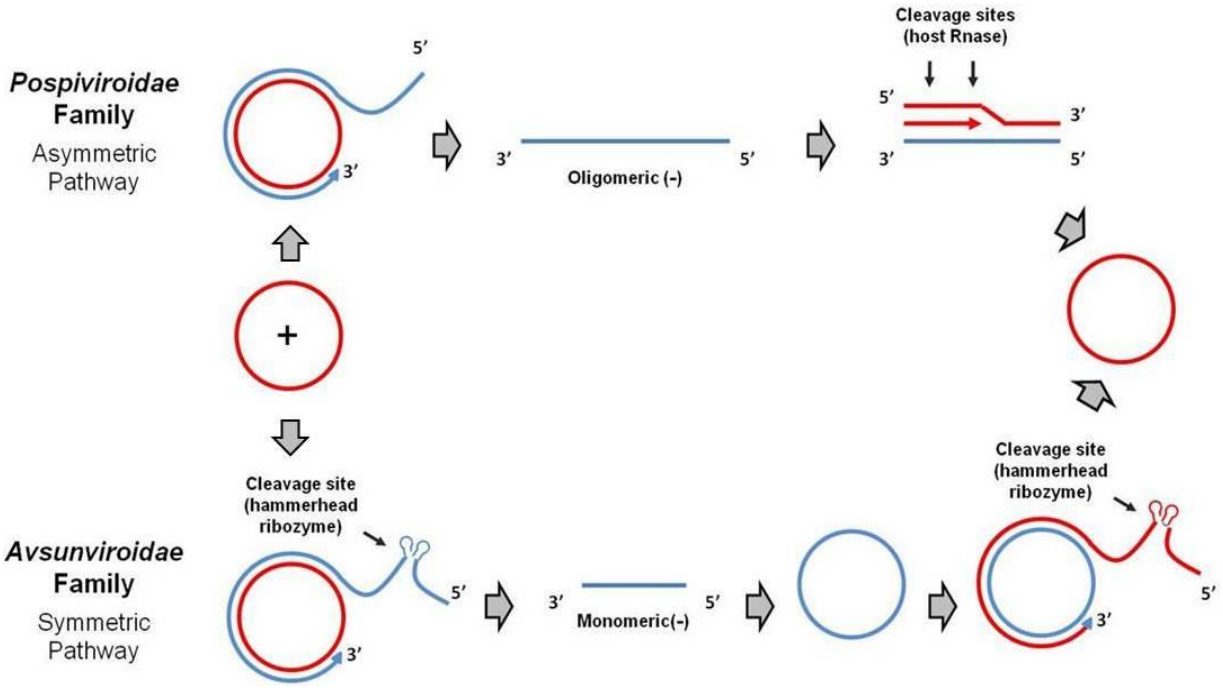


Figure 1.3: Replication cycle of the families *Pospiviroidae* and *Avsunviroidae* (Daròs *et al.* 2006). The difference between both pathways is the (−) template: the monomeric (−) circular RNA occurs in the so-called "symmetric pathway" of the *Avsunviroidae*, but not in the "asymmetric pathway" of the *Pospiviroidae*, where oligomeric (−) strands accumulate. Cleavage and ligation occur in (+) and (−) strands in the symmetric pathway with two rolling circles, but only in (+) strands in the asymmetric pathway with a single rolling circle. For the *Pospiviroidae* the three catalytic activities required are executed by a nuclear DNA-dependant RNA polymerase, RNase, and RNA ligase of the host. For the Avsunviroidae the cleavage is done by a hammerhead ribozyme motif of the viroid itself (Daròs *et al.* 2006).

The viroid progeny generated in the initially infected cells then invades adjacent cells through plasmodesmata using specific sequences or structural motifs within the viroid to finally reach the phloem (Ding *et al.* 1997). Consequently, viroids will move systemically to distal plant parts (Ding *et al.* 1997, Tsagris *et al.* 2008). After the description of a "pathogenicity domain" in PSTVd in the mid-eighties, it was hypothesized that symptom expression may be regulated by the ability of nucleotides within this portion of the molecule to interact with unspecified host components (Figure 1.4, Keese & Symons 1985, Owens & Hammond 2009, Palukaitis 2014). Indeed, it has been proposed that the mechanism of viroid pathogenesis is mediated directly by the viroid genome itself, or by viroid genome-derived ss or dsRNAs, and that expression of symptoms as a result of systemic infection may be an outcome of direct interactions of viroid-derived RNAs with unknown host factors (protein or nucleic acid), either in the organelle where the viroid replicates or in the cytoplasm where they accumulate during its movement (Flores *et al.* 2005).

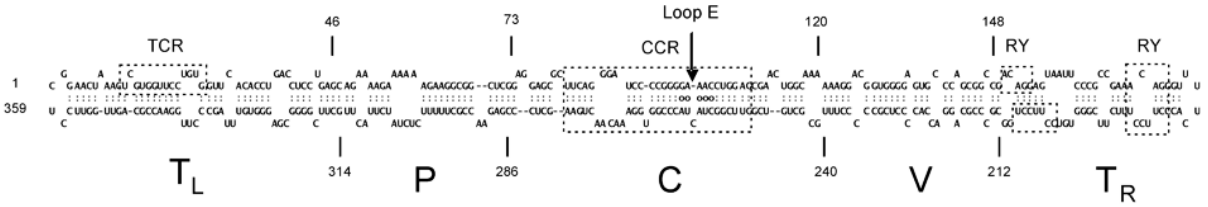


Figure 1.4: The rod-like secondary structure of PSTVd (intermediate strain) showing the five domains characteristic of members of the family *Pospiviroidae*: the Terminal Left (TL), Pathogenicity (P), Central (C), Variable (V), and Terminal Right (TR) (from Owens & Hammond 2009).

Over the years, many viroid-interacting host proteins have been discovered, such as DNA ligase 1, histones, a bromodomain-containing protein called "VIRP1/BRP1", the phloem-associated lectin "PP2" and many others (Katsarou *et al.* 2015). Evidence for direct interactions of viroids with specific host proteins has been generated by applying various techniques: e.g. by subjecting a cDNA expression library from viroid-infected leaves to an RNA ligand screening procedure (de Alba *et al*. 2003), by UV-irradition of infected leaves followed by tandem mass spectrometry analysis of cross-linked species (Daròs & Flores 2002), or by gel retardation analysis of ribonucleoprotein complexes (Gómez & Pallás 2001).

Furthermore, it was observed that viroid infection triggers the RNA silencing (or RNA-interference, RNAi) pathways of the host, resulting in large amounts of viroid small RNAs, called viroid small interfering RNAs (vd-siRNAs) of 21-24 nucleotides (Itaya *et al.* 2007). RNA-silencing is a powerful antiviral mechanism in plants and animals that involves the cleavage of a double-stranded RNA (dsRNA) into short RNAs by an enzyme Dicer that has RNase III domains (Baulcombe 2004). Vd-siRNAs may act as microRNAs (miRNAs) to downregulate the expression of physiologically important host genes and hence, induce disease symptoms (Wang *et al.* 2004). Itaya *et al.* (2007) showed that without possessing or triggering silencing suppressor activities, the PSTVd secondary structure plays a critical role in resistance to RNA-induced silencing complex (RISC)-mediated cleavage. Hence, these findings support the hypothesis that some infectious RNAs may have evolved specific secondary structures as an effective means to evade RNA silencing in addition to encoding silencing suppressor activities (Itaya *et al.* 2007). Additionally, this study showed that small RNAs of PSTVd produced in PSTVd-infected plants are incorporated into RISC and are functional in guiding sequence-specific cleavage of a target RNA. Therefore, these vs-siRNAs that are produced during viroid infection are biologically active in RNA silencing (Itaya *et al.* 2007).

Apart from RNA-silencing, host responses to viroid infection may also involve crosstalk between hormonal and defense-signaling pathways (Owens & Hammond 2009). To compare changes in gene expression and microRNA levels of PSTVd-infected and non-infected tomatoes, Owens *et al.* (2012b) used a combination of microarray and RNA sequence analysis. Significant changes were identified for a total of 19 genes involved in the biosynthesis or catabolism of different plant hormones like gibberellin, abscissic acid, brassinosteroids, cytokinin and jasmonic acid (Owens *et al.* 2012b).

In summary, there are three potential triggers for symptom induction: 1) viroid-protein interactions, 2) interactions with the RNA-silencing pathways of the host, 3) hormone-mediated responses (Figure 1.5; Navarro *et al.* 2012). However, many components of these pathways are still hypothetical and there is currently no agreement on a general mechanism for viroid pathogenesis.

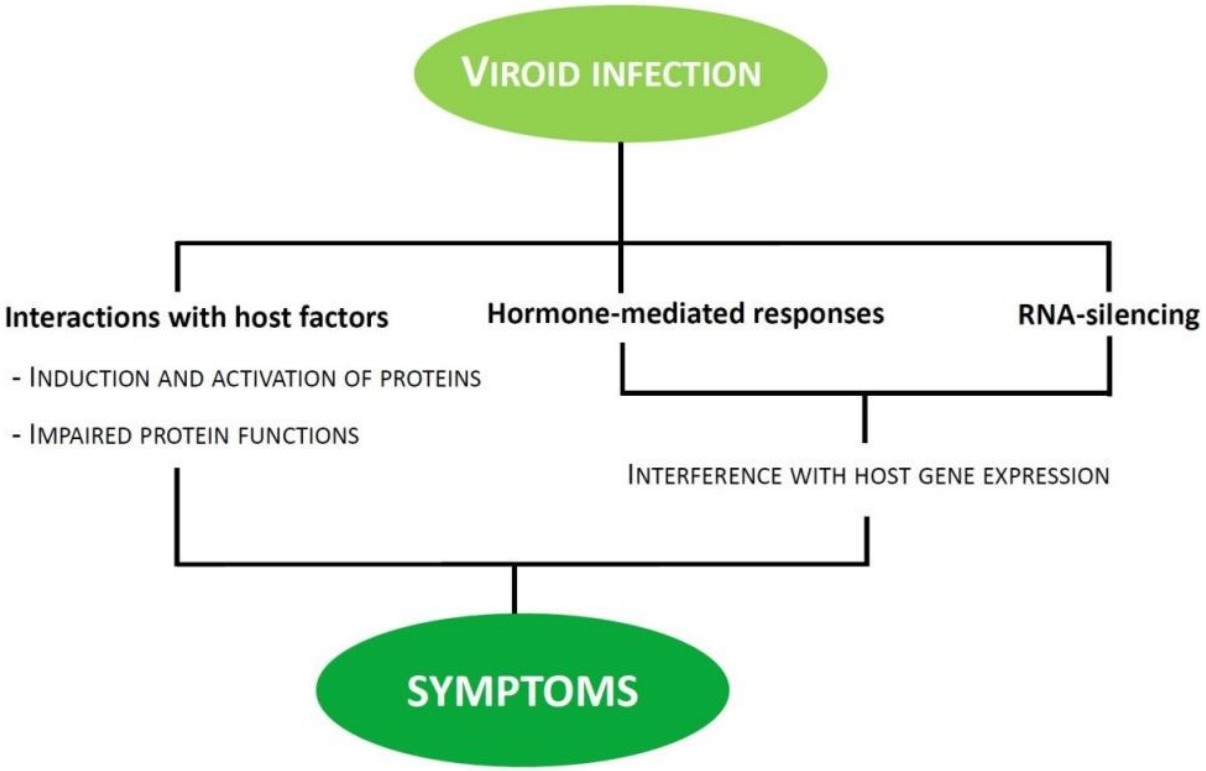


Figure 1.5: Potential pathways leading to the formation of macroscopic symptoms after a viroid infection (Modified from: Navarro *et al.* 2012).

1.4 DETECTION

Viroid control can best be implemented by using viroid-free starting material, since viroids spread very easily via mechanical ways (e.g. contact between plants, human handling, contaminated materials). Unfortunately, avoiding infection is not that straightforward since visual symptoms are often untrustworthy or absent (Mumford *et al.* 2000). Hence, diagnostic lab investigations are necessary to detect viroids in plants.

To establish a causative relationship between a viroid and a disease observed in a plant, four criteria - called "Koch's postulates" - need to be fulfilled (Figure 1.6). After isolation and identification of the viroid with molecular tests, the viroid needs to be inoculated into a healthy host plant (Figure 1.6). If the viroid can be re-isolated and verified to be identical to the originally found viroid, and if the produced symptoms are the same, then Koch's postulates are fulfilled (Figure 1.6).

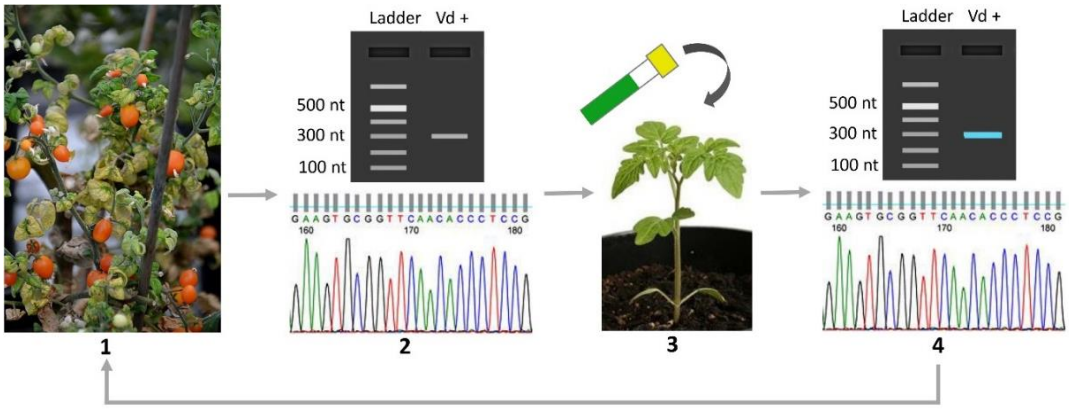


Figure 1.6: Schematic presentation of the fulfillment of Koch's postulates for a viroid (Vd) disease: 1) A plant is diagnosed with severe viroid-like symptoms, 2) Molecular tests (PCR, gel electrophoresis and sequencing) confirm that a specific viroid species is present, 3) The respective isolate is inoculated onto a healthy host, 4) Molecular confirmation of a viroid, and confirmation of the symptoms (grey arrow), which should be identical to the initial viroid found.

Since viroids do not encode for proteins, they cannot be detected using traditional immunological methods such as Enzyme Linked Immunosorbent Assay (ELISA) (Steger & Riesner 2003). Until some years ago Return-Polyacrylamide Gel-electrophoresis (R-PAGE) was a routinely used technique for viroid detection (Roenhorst *et al.* 2000). These days however, virologists usually rely on faster, user-friendly and more sensitive PCR-based techniques for standard detection of viroids: i.e. Reverse Transcription (RT-PCR) or Real-Time/Quantitative RT-PCR (RT-qPCR).

Both specific and generic RT-PCRs have been developed for viroids, but are insufficient for identification. For identification purposes traditional Sanger-sequencing is mostly used. Several validations of specific and generic pospiviroid detection tests have been published over the years (Boonham *et al.* 2004, Botermans *et al.* 2013, Monger *et al.* 2010, Olivier *et al.* 2014). In recent years, viroids have also been detected using next-generation sequencing (NGS) (Chiumenti *et al.* 2014, Fox *et al.* 2015).

Apart from PCR-based techniques, viroids can also be detected using tagged viroid-specific oligonucleotides (Salazar *et al.* 1992). For example, fluorescently tagged *in situ* hybridization probes (Figure 1.7) have been used to localize viroids on a subcellular-and tissue level in plants using confocal and (transmission/scanning) electron microscopy (Bonfiglioli *et al.* 1996).

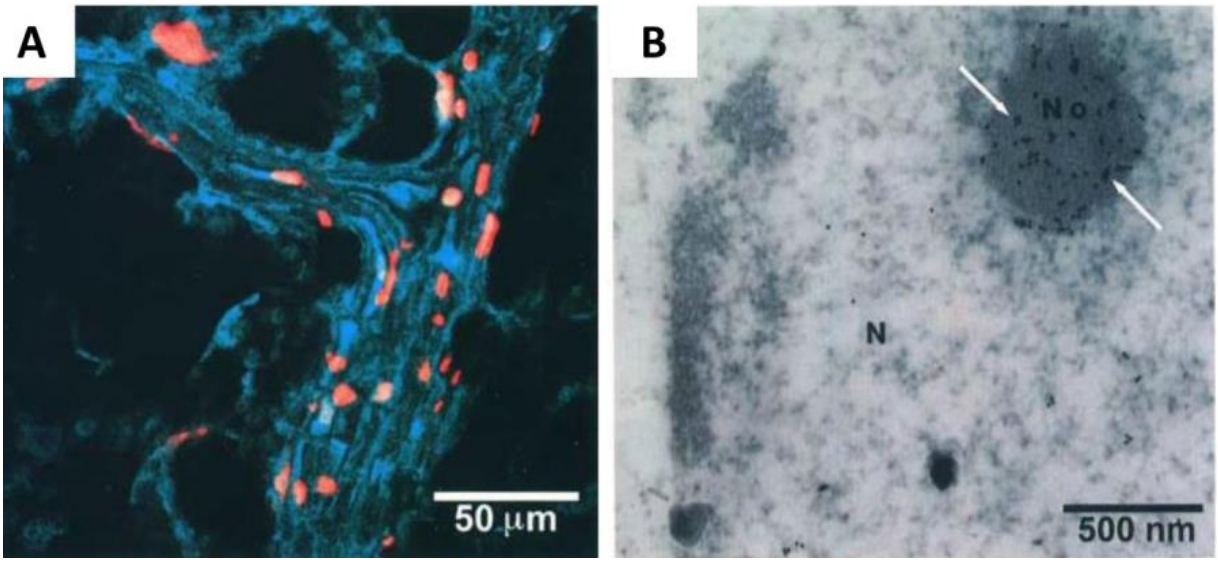


Figure 1.7: A) Confocal picture of vascular tissue of a CEVd-infected tomato leaf showing red/orange CEVd-signals; B) Transmission electron microscopy (TEM) picture of a CEVd-infected mesophyll tomato cell showing black (15nm gold) CEVd-signals (Bonfiglioli *et al.* 1996).

1.5 TRANSMISSION BY INSECTS

1.5.1 Plant virus transmission

Plant virus transmission through insect vectors is divided into three phases: acquisition, retention and inoculation (Pirone & Blanc 1996). For plant viruses, it was estimated that more than 80% are using arthropod vectors to move from one host to another (Fereres & Moreno 2009, Van den Heuvel *et al.* 1999). The large majority of these arthropods is of the insect order Hemiptera (Ng & Falk 2006). The best examples are aphids (*Aphidoidea*), whiteflies (*Aleyrodidae*), leafhoppers (*Cicadellidae*) and thrips (order *Thysanoptera*) (Van den Heuvel *et al.* 1999). Especially aphids and whiteflies seem to be very well adapted for virus transmission since their stylets recurrently pierce between plant cells to reach the phloem and/or to penetrate the actual cells without causing severe damage (Fereres & Moreno 2009). Gray & Banerjee (1999) reviewed the most important molecular and cellular mechanisms by which viruses are transmitted between plants. For plant viruses, a distinction is made between viruses with a *non-persistent* transmission, i.e. not retained by the insect vector for more than a few hours, and viruses with a *persistent* transmission, i.e. lifelong associated with the vector (Gray & Banerjee 1999). Non-persistent viruses are often called "*stylet-borne*" viruses, because they are carried on the mouthparts of vectors and are lost once a vector has fed on a host (Power 2000). Similarly, those viruses retained at the foregut have been called "*foregut-borne*" viruses (Nault & Ammar 1989). "*Cuticula-borne*" viruses, comprising both stylet and foregut-borne viruses, are those viruses that are carried on the cuticular lining of the vector feeding apparatus (Harris *et al.* 1996).

Persistently transmitted plant viruses are classified into *circulative* and *non-circulative* viruses, based on whether they are being actively internalized into the vector's hemocoel or not (Gray & Banerjee 1999). Circulative viruses can be further divided into *propagative* viruses, which replicate in their arthropod vector and addition to their plant host, and *non-propagative* viruses, which are replicating only in their plant hosts (Gray & Banerjee 1999).

1.5.2 Viroid transmission

The worldwide occurrence of viroids is clearly related to human activity, mainly in the form of international trade. Vegetative propagation of plants and trafficking of commercial crops have been the main contributors to the global spread of these minute plant pathogens. The European Food Safety Authority (EFSA) considers vegetative propagation of infected plant material to be the main source of viroid dispersal (EFSA Panel on Plant Health, 2011). In addition, mechanical contact between infected and non-infected plants and contamination with infected greenhouse materials play an important role (Verhoeven *et al.* 2010e). Additionally, evidence for seed and pollen transmission has been provided for various viroid species (Kryczynski *et al.* 1988, Singh & Dilworth 2009, van Brunschot *et al.* 2014). However, with regard to the transmission rate of pospiviroids by seeds, many discrepancies were reported (Faggioli *et al.* 2015). For example, studies focusing on PSTVd showed a variability in seed transmission rate from 0.3 % (van Brunschot *et al.* 2014) to 20 % (Kryczynski *et al.* 1988). In a recent study by Faggioli *et al.* (2015), more than 7000 seeds were collected from tomato plants that had been mechanically inoculated with TASVd, CEVd, CLVd and PSTVd. While all tested fruits and seeds reacted positive in RT-PCR, none of the seedlings were found infected by any of the studied pospiviroids (Faggioli *et al*. 2015). Therefore, the authors concluded that pospiviroid seed transmission in tomato is very rare (Faggioli *et al*. 2015). Lastly, also insects may play a role in viroid-transmission, either as direct vectors of viroids or as vectors of viruses in which viroids are encapsidated. This hypothesis of “transencapsidation” and insect-mediated transmission has received some, but not much, attention in the past in scientific studies (Francki *et al.* 1986, Querci *et al.* 1997, Salazar *et al.* 1995, Syller & Marczewski 2001). Transencapsidation can be defined as the encapsidation of the nucleic acids of a virus or viroid into the virion of another virus (Falk *et al.* 1995). Earlier, transencapsidation was observed frequently for different luteo- and potyviruses (Falk *et al.* 1995).

It has been hypothesized that the start of viroid epidemics in greenhouses is most commonly initiated by the presence of infected plants, with secondary spread being facilitated by mechanical transmission, or as speculated by Singh & Singh (1998) by insect activities. According to EFSA (2011), transmission of pospiviroids by aphids or bumblebees, within and between crops, has an unlikely to moderately likely probability rating. The high uncertainties on this assessment derive from the limited number of virus-viroid-host-vector combinations for which experimental data are available (EFSA Panel on Plant Health, 2011).

In early years, conflicting reports may have been caused by the use of inaccurate assays, different experimental designs, the use of visual readings, working in the field instead of in greenhouses, inaccurate detection of the viroid, contamination etc. (Schuman *et al.* 1980).

In 1980, Schuman *et al.* (1980) successfully established the actual presence of PSTVd in potato plants by means of a gel electrophoretic assay. This study evaluated the transmission of PSTVd by six common insect pests of potato, all yielding negative results (Table 1.3). De Bokx & Piron (1981) investigated PSTVd transmission between tomato plants by three aphid species: foxglove aphid (*Aulacorthum solani* Kaltenbach), potato aphid (*Macrosiphum euphorbiae* Thomas) and green peach aphid (*M. persicae*). As an inoculum source, infected tomato plants (cv. Sheyenne) and artificial diet solutions containing purified PSTVd were used. However, when allowing aphids to feed for 20 seconds on the parafilm membrane enclosing the artificial diet, it seemed that aphids did not feed as successfully as on detached tomato leaves. The results showed that only *M. euphorbiae* transmitted PSTVd in a non-persistent way (De Bokx & Piron 1981; Table 1.3). For another viroid, *Tomato planta macho viroid* (TPMVd) Galindo *et al.* (1986) showed highly efficient aphid transmission by *M. persicae*. On the other hand, the cow pea aphid (*Aphis craccivora* Koch) transmitted *Tomato apical stunt viroid* (TASVd) with a low efficiency (Walter, 1987, Table 1.3).

Several years later, various studies demonstrated the transmission of PSTVd between potato plants by the aphid *M. persicae* when the plants acting as inoculum source were co-infected with the viroid and *Potato leafroll virus* (PLRV) (Querci *et al.* 1997, Syller *et al*. 1997, Syller & Marczewski 2001). PLRV belongs to the genus of the luteoviruses and is known to be persistently transmitted by aphids (Goss 1930). Francki *et al.* (1986) had already shown that PSTVd-RNA can be transencapsidated by coat proteins of the *Velvet tobacco mottle virus* (VToMV). However, transencapsidation did not take place for *Potato virus Y* (PVY) (Singh *et al.* 1992a). In the experiments by Salazar *et al.* (1995), where plants were doubly infected with PLRV and PSTVd, 100% transmission of PSTVd was achieved. No transmission was observed when source plants were infected with only the viroid (Salazar *et al.* 1995). Following this research, Querci *et al.* (1997) allowed apterous aphids to feed on either singly (PSTVd) or doubly (PSTVd + PLRV) infected source plants. Then, after a transmission access period (TAP) of three days, aphids were transferred to young uninfected potato plants.

Inoculated plants were tested for PSTVd and/or PLRV after 15 and 45 days (after the TAP) by using a combination of nucleic acid spot hybridization (NASH) and enzyme-linked immunosorbent assay (ELISA) (Querci *et al.* 1997). Results showed that PSTVd was only detected in doubly infected plants, leading the authors to assume that transencapsidation of the viroid into the virus took place. To prove this hypothesis, different types of samples were treated before RNA extraction with Micrococcal Nuclease, a highly unspecific endo/exonuclease that was supposed to degrade non-encapsidated PSTVd RNA with a high efficiency. Samples treated with Micrococcal Nuclease and exhibiting PSTVd presence after PCR, illustrated that PSTVd had to be associated within the virus particle (Querci *et al.* 1997). The succesful transmission of PSTVd in conjunction with PLRV by aphids as reported by Querci *et al.* (1997) was later confirmed by Syller & Marczewski (2001, Table 1.3). The authors of this study also observed that symptoms in potato plants were much more severe in the case of mixed infections of PSTVd and PLRV compared with an infection with either pathogen alone (Syller & Marczewski, 2001).

Table 1.3: Overview of insect transmission studies with PSTVd, TASVd, TCDVd and TPMVd, showing + (positive) and – (negative) transmission results. T = transencapsidation. Studies between 1980-2012 are listed: (1) Schumann *et al.*, 1980, (2) De Bokx & Piron, 1981, (3) Francki *et al.* 1986, (4) Galindo *et al.*, 1986, (5) Walter, 1987, (6) Singh *et al.* 1992a, (7) Salazar *et al.,* 1995, (8) Querci *et al.*, 1997, (9) Syller *et al.* 1997, (10) Syller & Marczewski, 2001, (11) Antignus *et al.*, 2007, (12) Matsuura *et al.*, 2010, (13) Nielsen *et al.*, 2012.

Insect family	Species	Common name	PSTVd	TASVd	TCDVd	TPMVd
Aphididae	*Aphis craccivora*	Cowpea aphid		(5) -		
	Aulacortum solani	Foxglove aphid	(2) -			
	Macrosiphum euphorbiae	Potato aphid	(2) +			
	Myzus persicae	Green peach aphid	(1) -	(11) -		(4) +
			(2) -			
			(3) T+			
			(6) T-			
			(7) T+			
			(8) T+			
			(9) T+			
			(10) T+			
Aleyrodidae	*Bemisia tabaci*	Tobacco whitefly		(11) -		
Thripidae	*Frankliniella occidentalis*	Western flower thrip	(13) -			
	Thrips tabaci	Onion thrip	(13) -			
Apidae	*Apis mellifera*	Honeybee	(13) -			
	Bombus terrestris	Bumblebee	(13) -	(11) +	(12) +	
Other	*Empoasca fabae*	Potato leafhopper	(1) -			
	Leptinotarsa decemlineata	Colorado potato beetle	(1) -			
	Lygus Lineolaris	Tarnished plant bug	(1) -			
	Melanoplus femur-rubrum	Redlegged grasshopper	(1) -			
	Prodenia eridania	Southern armyworm	(1) -			

Transencapsidation and subsequent transmission through insects (such as aphids) can potentially have important epidemiological implications (Figure 1.8). A latently present viroid of a given crop can be incorporated into the capsid of a plant virus (e.g. into the icosahedral capsid of a *Luteovirus* sp.) and subsequently be transmitted by an insect vector (e.g. an aphid: Figure 1.8 A-B). This pathway of transencapsidation, followed by vector-mediated transport, can result in the infection of another host plant (Figure 1.8 C), and can reveal the viroid symptoms that were not expressed in the former host (Francki *et al.* 1986). In Figure 1.8 B, the viroid-virus association is acquired by an insect. However, the exact mechanism of how this acquisition and following survival and transmission of the pathogens occurs is not yet clear.

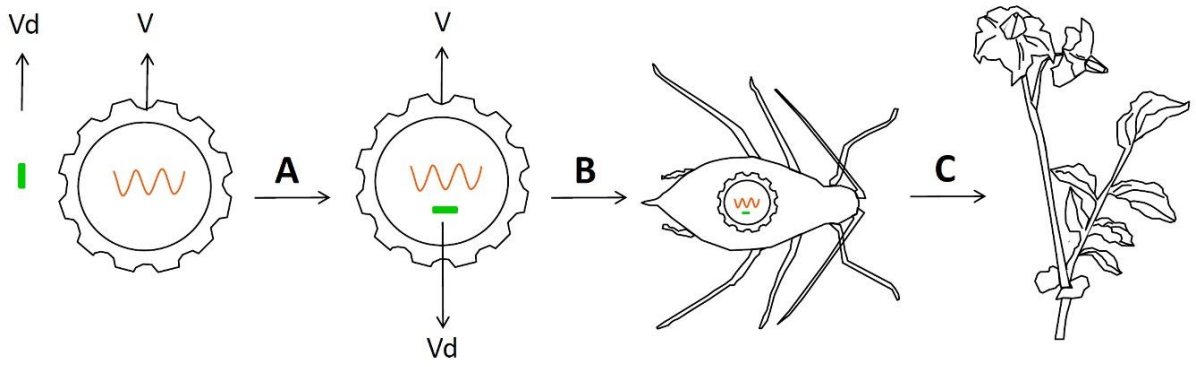


Figure 1.8: A: Viroid encapsidation (Vd) by a virus particle (V), B: Uptake by an aphid, C: Transmission to new host plants.

The research discussed above focused mainly on aphids as vectors of PSTVd. Over time, also other insect and viroid species have gained scientific attention. In 2007, Antignus *et al.* (2007) investigated transmission of TASVd by silver-leaf whiteflies (*B. tabaci*), green peach aphids (*M. persicae*) and bumblebees (*B. terrestris*). Whiteflies and aphids were introduced to TASVd-infected *Nicotiana rustica* (L.), *Physalis floridensis* (Rybd.), and tomato source plants for 48 h. Subsequently, they were transferred to individually caged healthy tomato plants for a 48 h-inoculation period and were tested for infection/contamination using Northern blot hybridization (Antignus *et al.* 2007). Bumblebees were introduced into a 50-mesh screenhouse, where some of the tomato plants had been mechanically inoculated with TASVd. This study concluded that no transmission of TASVd through (virus-free) *B. tabaci* and *M. persicae* took place (Antignus *et al.* 2007).

Surprisingly, *B. terrestris* appeared capable of transporting TASVd from infected to viroid-free tomato plants (Antignus *et al.* 2007). Therefore, the authors also suggested that transmission by bumblebees may be due to the wounding of the flowers during insect visits or by introducing infected pollen to the stigma of the flower. The vector-role of bumblebees in the transmission of viroids was also confirmed by Matsuura *et al.* (2010) through experiments in greenhouses. Bumblebees (*Bombus ignitus*) were introduced in the greenhouses after mechanical inoculation of tomato plants with TCDVd (Matsuura *et al.* 2010). After more than one month, TCDVd was detected by qPCR in the non-infected plants (Matsuura *et al.* 2010). The authors suggested that TCDVd is mechanically transmitted with crude sap via the insect mandibles. However, one should also consider horizontal transmission through viroid-contaminated pollen carried by bumblebees.

Nielsen *et al.* (2012) could not confirm the results of Antignus *et al.* (2007) and Matsuura *et al.* (2010). The latter study explored the transmission of PSTVd by thrips (*Frankliniella occidentalis* and *Thrips tabaci*), honeybees (*Apis mellifera*) and bumblebees (*B. terrestris*). Both intra- and interspecies transmission between ornamentals and vegetable crops of the *Solanaceae* were investigated, but no insect-mediated transmission was recorded (Nielsen *et al.* 2012). The authors emphasized that transmission of PSTVd by transencapsidation in PLRV particles was not considered in their experimental design, but should certainly be taken into account in the future (Nielsen *et al.* 2012).

1.6 DISTRIBUTION

During the last decade, most of the European viroid research focused on the family of the *Pospiviroidae*. Pospiviroids are known to infect important economic crops, such as tomato (*S. lycopersicum*) and potato (*Solanum tuberosum*, L.), inducing symptoms that vary with viroid strain, plant variety and climatic conditions, but are generally characterized by reduced growth and chlorosis of the leaves (Hadidi *et al.* 2003). During the past decade, however, various European surveys have revealed that many members of the family *Pospiviroidae* have also been *latently* present in ornamental plants belonging to the *Solanaceae* family (Di Serio 2007, Luigi *et al.* 2011, Verhoeven *et al.* 2008a-b, Verhoeven *et al.* 2010b). Examples of latently infected ornamentals are *Cestrum* spp. L. (jessamines), *S. jasminoides* (jasmine nightshade), *Lycianthes rantonnetii* Carrière Bitter (blue potato bush), *Brugmansia* spp. Pers (angel's trumpets), *Verbena spp.* L. (vervain), *Streptosolen jamesonii* Benth. Miers (marmalade bush), *Petunia spp.* Juss., *Vinca minor* L. (lesser periwinkle) and *Dahlia spp.* Cav. (Bostan *et al.* 2004, Luigi *et al.* 2011, Singh 2006, Singh & Baranwal 2006, Shiraishi *et al.* 2013; Torchetti *et al.* 2011, Verhoeven *et al.* 2007b, Verhoeven *et al.* 2008, Verhoeven *et al.* 2010a-b, Verhoeven *et al.* 2013). These asymptomatic viroid-infected plants can act as reservoirs from which viroids may spread to cultivated species and induce diseases (Singh 2006a-b, Verhoeven *et al.* 2010c).

In Belgium, two large outbreaks of PSTVd and *Columnea latent viroid* (CLVd) were reported in tomato in 2006 (Verhoeven *et al.* 2007a). To investigate pospiviroids in Belgium more extensively, three Belgian agricultural research institutes (ILVO, CRA-W and Scientia Terrae) conducted a national project TOPOVIR ("Transmission Of Pospiviroids") in the period 2009-2011 focussing on the transmission risk from ornamentals to vegetable crops. In 2009, the project FYQUARSTAT project focused on TASVd and CSVd, for which ILVO and CRA-W conducted surveys in tomato, ornamentals and chrysanthemums. Both institutes continued their research within the EUPHRESCO ERA-net project DEP2 ("Detection and Epidemiology of Pospiviroids") in 2012, together with 7 other European partners. During this last project, ILVO focused especially on the transmission of viroids via insects and to a lesser extent seeds (Faggioli *et al.* 2015).

Table 1.4 shows 11 complete genome pospiviroid sequences that were obtained during surveys conducted in the framework of the TOPOVIR, EUPHRESCO and DEP2 projects or that were found in samples sent to the Diagnostic Centre for Plants (DCP-ILVO) by growers. Table 1.4 shows that several of these pospiviroids were found in asymptomatic ornamental plants like Vinca sp., *L. rantonnettii* and *S. jasminoides.*

Table 1.4: Overview of pospiviroid isolates found in Belgium during surveys or in samples sent by growers to the Diagnostic Centre for Plants (DCP, ILVO) in the period 2009-2015. Year, Latin and Common Name of the host species in which each viroid was found and the GenBank Accession Number (N^{O}) are presented. To identify which pospiviroid species caused each infection, generic and specific PCR-tests and Sanger dideoxy sequencing were performed.

Year	Latin Name	Common Name	Pospiviroid	GenBank Accession N°
2009	*Chrysanthemum*	Chrysanth	CSVd	KX084709, KX084710
2010	*Lycianthes rantonnetii*	Blue potato bush	TASVd	KF484879
	Solanum jasminoides	Jasmine nightshade	PSTVd	KU714935
	Petunia sp. Hot Pink	Petunia	TCDVd	KU714936
	Solanum lycopersicum	Tomato	TCDVd	KU714937
2011	*Solanum jasminoides*	Jasmine nightshade	CEVd	KX084708
2012	*Solanum jasminoides*	Jasmine nightshade	TASVd	KF484878
2014	*Chrysanthemum*	Chrysanth	CSVd	KX084711, KX084712
2015	*Vinca sp.*	Periwinkle	TCDVd	KU714934

Recently, the cocadviroid *Hop latent viroid* (HLVd) was found for the first time in Belgian hop plants (*Humulus lupulus*, L.) (De Jonghe *et al.* 2016). In hop production, it seems that the majority of hop fields worldwide are infected with HLVd (Jakse *et al.* 2015). HLVd does not cause any visual symptoms in hop but does result in a serious decrease in the content of alpha acids, which are important for the beer industry (Matoušek *et al.* 2001). In Slovenian hop gardens, *Hop stunt viroid* (HSVd) and *Citrus bark cracking viroid* (CBCVd) have recently been discovered in numerous severely affected plants (Jakse *et al.* 2015). HSVd and CBCVd have not (yet) been found in Belgian hop gardens during surveys.

In 2005, *Petunia* hybrid plants coming from the USA were inspected after entering the post-entry quarantine station of the Plant Protection Service (PPS) in the Netherlands (Verhoeven *et al.* 2007b).

PPS found TCDVd to be present; this was the first report of this viroid in *P. hybrida* (Verhoeven *et al.* 2007b). In 2006, CEVd was detected in *Verbena* sp., PSTVd in *Brugmansia* sp. and *S. jasminoides* and TASVd in *Cestrum* sp. (Verhoeven *et al.* 2008a). Subsequently, CEVd and TASVd were also identified in *S. jasminoides* (Verhoeven *et al.* 2008b), and more pospiviroid infections were reported in plant species from the families *Gesneriaceae* (*Nematanthus* sp.), *Verbenaceae* (*Verbena* sp.) and *Apocynacea* (*Vinca* sp.) (Verhoeven *et al*. 2012). Additionally, Verhoeven *et al.* (2010d, 2012) indicated that ornamental species may act as inoculum sources of pospiviroid outbreaks in tomato.

In the UK, the first report of a PSTVd outbreak in commercial tomatoes dates back to 2003 (Mumford *et al.* 2003). Before, PSTVd had only been found under controlled conditions in a UK potato germplasm collection (Cammack & Richardson 1963). At least for PSTVd, worldwide distribution can now be assumed. In Peru, PSTVd has been detected in avocado (*Persea Americana*, Mill.), where infections often remain latent, unless the tree is co-infected with ASBVd (Querci *et al.* 1995). In New Zealand PSTVd was reported to be associated with a new disease of glasshouse tomato and *Capsicum* crops (Lebas *et al.* 2005). In conclusion, new reports of pospiviroid members are emerging in all corners of the world.

1.7 REGULATORY STATUS OF POSPIVIROIDS IN THE EU

Of all *Pospiviroidae* species occurring in the EU, only two have been listed as regulated pests: CSVd and PSTVd. CSVd is listed on Annex II Part A Section II of the Directive 2000/29/EC as a harmful organism whose introduction into and spread within EU member states needs to be banned in *Dendranthema* sp. species intended for planting, other than seeds. PSTVd is listed on Annex I Part A Section I of the Council Directive 2000/29/EC as a harmful organism and hence its introduction into (and spread within) all EU member states is prohibited. In 2007 the European Commission (EC) also adopted emergency measures to further prevent the introduction into, and the spread within, the EU territory of PSTVd (Commission Decision 2007/410/EC). This decision defines measures for plants belonging to the genera *Brugmansia* and of the species *S. jasminoides*, intended for planting, including seeds. Additionally, this Decision describes measures for import and movement of the specified plants within the EU territory and requires the EU member states to conduct official surveys and to notify the results to the Commission.

In Belgium, it was observed that, since the implementation of this Decision and the replacement of new mother material by the growers, PSTVd was much less detected in certain host plants (such as *S. jasminoides* and *Brugmansia*, Olivier *et al.* 2012). Instead, it seemed that PSTVd was being replaced by other (non-quarantine regulated) viroids like TASVd and CEVd (Olivier *et al.* 2012). Also in the Netherlands, the infection rate of PSTVd decreased substantially since the eradication measures of the EC (Verhoeven *et al.* 2012). Only recently, in May 2015, the Decision 2007/410/EC was repealed (2015/749).

Besides the obligatory EU-regulation, PSTVd and CSVd can also be found on the “A2-list” of the “European and Mediterranean Plant Protection Organization” (EPPO). EPPO recommends its member countries to regulate A2-listed pests as quarantine organisms. TASVd and CBCVd are currently the only viroids to be included on the EPPO “Alert List”, indicating that these pathogens could possibly present a risk to EPPO member countries (EPPO 2016, https://www.eppo.int). TASVd has been on this list since 2003, while CBCVd was added only recently (2015).

Chapter 2:

The role of weeds in the epidemiology of pospiviroids

Modified from: Van Bogaert, N., Smagghe, G., and De Jonghe, K. (2015). The role of weeds in the epidemiology of pospiviroids. *Weed Research*. 55(6), 631-638.

2.1 INTRODUCTION

Pathogens that attack crops can often survive on suitable alternative weed hosts when the crop is absent, providing a reservoir from which infection of the crop can occur (Wisler & Norris 2005). The extent to which these weeds may actually contribute to (re)establishment of diseases in crops, is a major issue in integrated pathogen management (Wisler & Norris 2005). For viroids, several solanaceous weed species, such as *Solanum nigrum* L. (black nightshade) and *Solanum dulcamara* L. (woody nightshade), have been identified as experimental hosts (EFSA Panel on Plant Health, 2011). Other solanaceous (wild) plants such as *Solanum luteum* Mill. (woolly nightshade) and *Lycium barbarum* L. (Chinese wolfberry) have been proposed as potential experimental hosts (EFSA Panel on Plant Health, 2011).

In the beginning of the 1970s, several plant species belonging to the *Amaranthaceae, Boraginaceae, Campanulaceae, Caryophyllaceae, Compositae, Convolvulaceae, Dipsaceae* and *Nolanaceae* were mechanically inoculated with viroid-infected plant material and in the following weeks emerging symptoms were assessed (O'Brien 1972, Singh 1973). However, the results from these studies must be interpreted with care since diagnostics were based on symptom development without molecular confirmation tests, which were not available at the time. Since then, only a few studies have focused on the role of weeds as inoculum reservoir for viroids. Antignus *et al.* (2007) sampled 19 weed species from the field and inoculated them using carborundum and *Tomato apical stunt viroid* (TASVd)-infected tomato sap. None of these weeds were found to be host of TASVd (Antignus *et al.* 2007).

In another study, weeds typical for potato and hop fields were biolistically inoculated with mixtures of *Hop stunt viroid* (HSVd) and PSTVd (Matoušek *et al.* 2007). During the biolistic inoculation of viroids, viroid RNA or cDNA is coated onto golden microcarriers (1µm) and consequently bombarded into the plant cells using a Helios Gene Gun (Bio-Rad) (Matoušek *et al.* 2007). Both RNA as well as cDNA inocula were prepared in the expectation that this experimental approach would increase the chance of identifying potential hosts among analyzed weed plants (Matoušek *et al.* 2007). Twenty-six days post-inoculation, *Chamomilla recutita* L. (weed chamomile) and *Anthemis arvensis* L. (corn chamomile) were identified as new experimental hosts (Matoušek *et al.* 2007). In addition to these putative hosts, a number of plants also gave faint positive results after reverse transcription (RT)-PCR: i.e.: *Galinsoga ciliata* L. (hairy galinsoga) and *Amaranthus retroflexus* L. (redroot pigweed).

Also the parasitic weed *Phelipanche ramosa* L. Pomel (hemp broomrape) has been reported as an experimental host plant for PSTVd (Ivanova *et al.* 2014). Recently Mackie *et al.* (2016) reported the detection of PSTVd in volunteer crop plants of tomato, pepper and chilli and various introduced weed species of the families of the *Solanaceae*, *Asteraceae* and *Chenopodiaceae* in Western Australia.

In 2010 ten *S. jasminoides* plants were collected from one grower in the framework of the project TOPOVIR ("Transmission of Pospiviroids") and tested for viroid infection by ILVO. All plants were found positive for PSTVd and were kept as inoculum source for experiments in a growth chamber. During the following weeks, several weed species started growing spontaneously in the pots. These weeds were *S. oleraceus*, *Betula pendula* Roth (silver birch), *Stellaria holostea* L. (greater stitchwort) *Stachys recta* L. (stiff hedgenettle), *Senecio vulgaris* L. (common groundsel) and several *Poaceae* spp. (true grasses). The weeds were in direct root and leaf contact with the infected *S. jasminoides*. After testing a leaf sample of these plants for viroid presence, *S. oleraceus*, *S. recta*, *Poa trivialis*, *S. vulgaris* and *B. pendula* tested positive (De Jonghe *et al*. 2012, TOPOVIR 2011).To exclude direct external leaf contamination of the plants, the above ground parts of the *S. jasminoides* source plants were removed and the weeds were left to grow. Although no symptoms were recorded, newly formed leaves of *S. oleraceus*, *S. recta* and *B. pendula* were confirmed positive for PSTVd after sequence analysis. Due to these ad hoc findings, further research on weed hosts was initiated.

The objective of this Chapter was to investigate whether commonly occurring weed species are potential hosts of pospiviroids in Belgium. This information is important to estimate the transmission risk from potential weed reservoirs to economically important crops, such as tomato and potato, where viroids can cause severe disease. Three different approaches were used to meet the objective. First, weeds growing inside (or in the vicinity) of pots of ornamental viroid hosts in commercial greenhouses were sampled during a survey and tested for viroids. Second, an experiment was set up in a polytunnel greenhouse to test whether weeds growing spontaneously, and in close contact with infected ornamental plants, would also become infected. Third, six commonly occurring weed species were mechanically inoculated with TASVd and tested after six weeks. The outcomes of this study are discussed together with results from literature to present a comprehensive view of the subject.

2.2 MATERIAL AND METHODS

To investigate the role of weeds as potential reservoirs of pospiviroids, one survey, two independent greenhouse experiments and one mechanical inoculation experiment were performed. Below, these three types of experiments are described in detail.

2.2.1 Survey for weed plants in ornamental greenhouses

2.2.1.1 Sampling procedure

The weed survey was performed in two ornamental greenhouses in the East-and West-Flanders provinces of Belgium. From each sampled plant of the solanaceous crop, three leaves from stem to top were collected in Ziplock® bags, labeled and kept at 4°C before analysis. The same was done for weeds growing in close proximity to ornamentals (within the pots and outside of them).

2.2.1.2 Detection of pospiviroids

In the lab, a composite sample of 100 mg of each plant was put in an Eppendorf tube and frozen in liquid nitrogen. After crushing the plant material, total RNA was extracted using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA) and cDNA was synthesized using the iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). The pospi1 FW/RE and Vid FW/RE-primers were used for a general pospiviroid RT-PCR detection (Verhoeven *et al.* 2004, Table 2.1). The samples were analyzed using capillary electrophoresis (QIAxcel Advanced System, Qiagen, Hilden, Germany) and positive amplicons were sent for sequencing (Macrogen Europe, Amsterdam, the Netherlands). When weak or ambiguous signals were detected during electrophoreses, viroid RNA was re-analyzed using an RT-qPCR with the Agpath-ID™ one-step RT qPCR Kit (Applied Biosystems, Foster City, CA, USA) using the generic primers and probes of Botermans *et al.* (2013) or the specific primers and probes of Boonham *et al.* (2004) and Monger *et al.* (2010) in separate assays (Table 2.1). Based on validation data, Cq-values higher than 35 were considered as negative (Table 2.2).

Table 2.1: Name, type/orientation, sequence and reference of the different primers and probes that were used for RT-qPCR detection of pospiviroids.

Name	Type/Orientation	Sequence (5'-3')	Reference
Pospi1-FW	FW	GGGATCCCCGGGGAAAC	Verhoeven *et al.* 2004
Pospi1-RE	RE	AGC TTCAGTTGT(T/A)TCCACCGGGT	
Vid-FW	FW	TTCCTCGGAACTAAACTCGTG	
Vid-RE	RE	CCAACTGCGGTTCCAAGGG	
TCR-F 1-1	FW	TTCCTGTGGTTCACACCTGACC	Botermans *et al.* 2013
TCR-F 1-3	FW	CCTGTGGTGCTCACCTGACC	
TCR-F 1-4	FW	CCTGTGGTGCACTCCTGACC	
TCR-F PCFVd	FW	TGGTGCCTCCCCCGAA	
TCR-F IrVd	FW	AAT GGTTGCACCCCTGACC	
TR-R1	RE	GGAAGGGTGAAAACCCTGTTT	
TR-R CEVd	RE	AGGAAGGAGACGAGCTCCTGTT	
TR-R6	RE	GAAAGGAAGGATGAAAAT CCTGTTTC	
pUCCR	Probe (6FAM/MGB)	CCGGGGAAACCTGGA	
PSTV-251T	Probe	CAGTTGTTTCCACCGGGTAGTAGCCGA	Boonham *et al.* 2004
PSTV-231	FW	GCCCCCTTTGCGCTGT	
PSTV-296	RE	AAGCGGTTCTCGGGAGCTT	
CLVd-F	FW	GGTTCACACCTGACCCTGCAG	Monger *et al.* 2010
CLVd-F2	FW	AAACTCGTGGTTCCTGTGGTT	
CLVd-R	RE	CGCTCGGTCTGAGTTGCC	
CLVd-P	Probe (6FAM/BHQ1)	AGCGGTCTCAGGAGCCCCGG	
TASVd-P2-228	Probe (6FAM/TAMRA)	TCTTCGGCCCTCGCCCGR	
TASVd-F2-200	FW	CKGGTTTCCWTCCTCTCGC	
TASVd-R2-269	RE	CGGGTAGTCTCCAGAGAGAAG	

Table 2.2: Validation results for one-step RT-qPCR tests (Bio-Rad CFX96 Touch™ qPCR, Hercules, CA, USA) of a TASVd and a CLVd dilution series (10^{-1}-10^{-8}) after RNA-extraction of a TASVd and a CLVd-infected tomato plant (*S. lycopersicum*, L. cv. Marmande) and amplication using the primers of Botermans *et al.* (2013). The dilution series was tested in duplo and Mean Cq-values ± Standard Deviation (SD) are presented. In each assay, all samples were also amplified using the plant internal positive control COX (F/R)-primers, in conjunction with positive and negative controls (results not shown). The assays were executed by three different people: Sébastien Morio ("SM"), Inge De Roo ("IDR") and Shana Vandierendonck ("SV") of the Diagnostic Centre for Plants (DCP, ILVO) in order to test the reproducibility of the detection results.

Dilution series	**SM**				**IDR**		**SV**	
	TASVd		**CLVd**		**TASVd**		**CLVd**	
	Mean Cq	SD	Mean Cq	SD	Mean Cq	SD	Mean Cq	SD
No dilution	18.7	0.12	/	/	18.2	0.69	20.1	0.06
10^{-1}	20.5	0.23	23.3	0.13	20.1	0.03	23.4	0.02
10^{-2}	23.1	0.04	26.7	0.09	22.6	0.05	26.8	0.12
10^{-3}	26.4	0.04	29.5	0.10	26.0	0.18	29.4	0.06
10^{-4}	30.1	0.08	32.2	0.15	29.3	0.06	32.2	0.05
10^{-5}	33.7	0.15	36.5	2.37	32.5	0.01	35.8	1.12
10^{-6}	37.1	1.24	No detection		35.3	0.54	No detection	
10^{-7}	39.0	0.38	No detection		No detection		No detection	
10^{-8}	No detection		No detection		No detection		No detection	
10^{-9}	No detection		No detection		No detection		No detection	
NTC	No detection		No detection		No detection		No detection	

2.2.2 Greenhouse experiments with infected host plants and weeds

2.2.2.1 Experimental design

Five TASVd-infected *S. lycopersicum* (cv. "Marmande") seedlings were planted in the soil of a tunnel greenhouse. The soil in this greenhouse had been tilled before the start of the experiment. In the following weeks, various weeds started growing spontaneously. The same experiment was repeated in a separate plot of the greenhouse, with five TASVd-infected *S. jasminoides* plants (Figure 2.1).

Figure 2.1: Experimental design of the greenhouse contact experiment with five TASVd-infected *S. jasminoides* with ±50cm distance between each other, A: start of the experiment, B: after six weeks.

2.2.2.2 Sampling procedure

After 4, 6 and 8 weeks samples were taken of weed species that had grown spontaneously from the soil seed bank and that were in close physical contact with the viroid-infected host plant. A total of 25 weed plants was sampled during both experiments (i.e. *S. lycopersicum* and *S. jasminoides* experiment) 4 weeks after the start of the experiment. These weed plants were individually labelled and re-tested after 6 and 8 weeks. While sampling the weed species, each leaf sampling was performed with a new set of gloves and sterile plastic bags. Two leaf samples of each individual weed plant were taken, i.e.: one leaf that physically touched the infected host plant ("C") and one leaf that was not in contact with the infected host plant ("NC").

This was done to avoid false positives due to physical/mechanical contamination of the weed plant and to be certain of the systemic spread of the viroid in this plant. The five TASVd-infected *S. jasminoides* and tomato plants were also tested individually during each testing trial.

2.2.2.3 Detection of pospiviroids

From each of the sampled leaves, RNA was extracted and cDNA-synthesis and RT-PCRs were performed (identical as described in "2.2.1.2 Detection of pospiviroids").s

2.2.3 Viroid detection in six weed species after mechanical inoculation with carborundum

2.2.3.1 Experimental design

Six weed species, commonly found in the vicinity of tomato and ornamental greenhouses and potato fields, were selected for inoculation: *Rumex crispus* (curly dock), *Chamomilla recutita* (chamomile), *Sonchus oleraceus* (common sowthistle), *Galinsoga parviflora* (potato weed), *Stellaria media* (common chickweed) and *Chenopodium album* (common lamb's-quarters). The inoculum source was TASVd (GenBank-AN: KF484878) maintained on *S. jasminoides* and *S. lycopersicum* (cv. "Marmande"). This species was selected because of three reasons: 1) it is one of the most prevalent found pospiviroid in ornamental *Solanaceae* in Belgium, 2) it causes similar symptoms as PSTVd and TCDVd in susceptible crops like tomato and potato and shares a similar host range with these viroids, 3) despite its presence on the EPPO Alert List since 2006, it is not quarantine-regulated which implies less stringent working conditions.

Ten plants of each weed species were inoculated at a young stage (3-6 leaves). During each inoculation trial, two plants of three viroid host species were also inoculated as a technical control: potato (*S. tuberosum*), eggplant (*Solanum melongena*, L.) and chilli pepper (*Capsicum annuum*, L.).

2.2.3.2 Inoculation procedure

For preparing the inoculum, 1 g of leaf tissue of the TASVd-infected maintenance hosts was brought into extraction bags (Bioreba, Reinach, Switzerland) with 10 ml of ice-cold inoculation buffer (0.01 M potassium phosphate buffer, pH 7 and 0.2% sodium sulfite) and consequently the plant material in the bags was crushed using a Homex6 homogenizer (Bioreba, Reinach, Switzerland). The filtered mixture was collected with a pipette and divided over 1.5 ml-Eppendorf tubes kept on ice. A cotton swab was dipped in carborundum (silicium carbide, size C500, Department Physics and Astronomy, Ghent University) and consequently used to rub the infected plant mixture along the midrib of the leaf. Three leaves of each plants were treated and then marked with plastic tags (2x1cm) that were folded around each leaf. A few seconds after inoculation, plants were rinsed thoroughly with distilled water. During the following weeks, plants were inspected visually and abnormalities were recorded.

2.2.3.3 Sampling and testing of the plants

Six weeks post-inoculation five to six newly formed leaves of each plant were sampled, avoiding the tagged leaves. RNA-extraction, cDNA-synthesis and RT-PCR were conducted on those samples as described above.

2.3 RESULTS

2.3.1 Survey for weed plants in ornamental greenhouses

In the period 2012-2014 eight samples of weed species that were in physical contact with viroid-containing solanaceous hosts, were collected at two commercial greenhouses in West- and East-Flanders, Belgium. These weeds belonged to the families of the *Caryophyllaceae*, *Geraniaceae* and *Onagraceae*. After testing them for the presence of pospiviroids using the generic Pospi1FW-RE primers (Verhoeven *et al.* 2004), none of these were positive (Table 2.3). In contrast, samples of *S. jasminoides* and *Verbena x hybrida* collected at these locations were infected with pospiviroids (Table 2.3). Sequence analysis confirmed the presence of TASVd in *S. jasminoides* and *Citrus exocortis viroid* (CEVd) in *Verbena x hybrida*.

Table 2.3. Family, scientific and common name of the plant species collected during surveys at two commercial greenhouses. The number of positive plants on the total collected per species is presented in the last column.

Family	Genus/species	Common name	Positive/Collected
Caryophyllaceae	*Stellaria media*	Chickweed	0/3
Geraniaceae	*Pelargonium* sp.	Geranium	0/4
Onagraceae	*Epilobium* sp.	Willowherbs	0/1
Solanaceae	*Brugmansia* sp.	Angel's trumpet	0/4
	Petunia sp.	Petunia/Surfinia	0/6
	Solanum jasminoides	Jasmine	11/11
Verbenaceae	*Verbena x hybrida* "Lanai white blancena"	Verbena	1/1

2.3.3 Greenhouse experiments with infected host plants and weeds

During the contact experiments, we sampled the following weed plants that were in close proximity to the TASVd-infected *S. lycopersicum* and *S. jasminoides*: *Amaranthus retroflexus* L.*, Sonchus oleraceus* L.*, Galinsoga parviflora* Cav.*, Taraxacum officinale* F.H.Wigg.*, Stellaria media* L. Vill. *Echinochloa crus-galli* L. P. Beauv. and *Polygonum persicaria* Gray (Table 2.4). After capillary electrophoresis it was observed that several leaf samples gave weak positive bands (Figure 2.2). However, a weed plant was only considered infected if the two separately taken leaf samples (i.e. "C" and "NC") both tested positive after the different testing trials through time (4, 6 and 8 weeks, Table 2.4).

Table 2.4. Detailed test results of the 5 samples of 7 different weed species collected after 4, 6 and 8 weeks during the greenhouse experiments with the 5 TASVd-infected plants of *S. lycopersicum* and *S. jasminoides*. The number of positives on the total number of "C" (white) or "NC" (grey) leaf samples are presented. * indicate that these test results were obtained from the same *E. crus-galli* plant.

Experiment	Week	*A. retroflexus*		*S. oleraceus*		*G. parviflora*		*T. officinale*		*S. media*		*E. crus-galli*		*P. persicaria*	
1. *S. lycopersicum*	4	0/5	0/5	2/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5
	6	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	8	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5
2. *S. jasminoides*	4	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5	1/5*	1/5*	0/5	0/5
	6	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	8	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

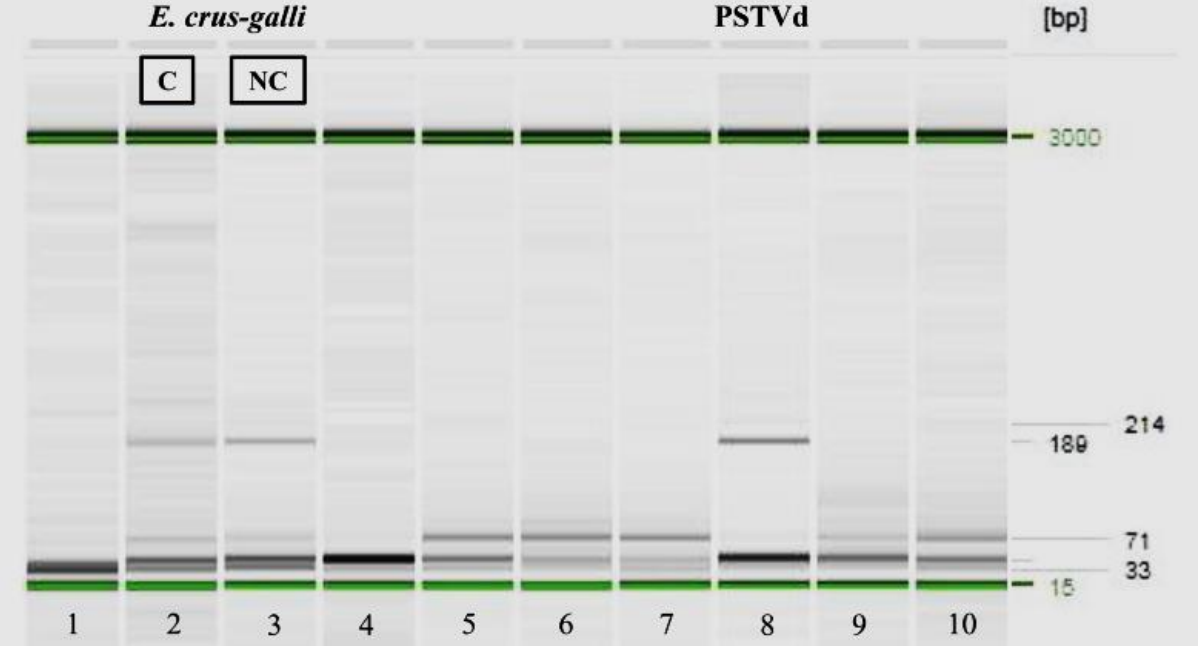


Figure 2.2: Capillary electrophoreses output after a pospi1-RT-PCR (Verhoeven *et al.* 2004) conducted after four weeks. Positive amplification results at ±200nt can be observed for *E. crus-galli* in column 2, containing the leaf-sample that was in contact with *S. jasminoides* ("C") and in column 3, which contains material from the leaf sample that was not in contact with *S .jasminoides* ("NC"). Column 8: positive PSTVd control. Columns 1, 4-7 = other sampled weed species that tested negative. Column 9-10 = no template controls (ntc).

This observation was only found once, namely for *E. crus-galli* in the *S. jasminoides* contact experiment after week 4 (Figure 2.2, Table 2.4). However, after 6 and 8 weeks these plants did not test positive anymore. None of the other *E. crus-galli* plants resulted in a simultaneous "C" and "NC" positive detection (Table 2.4). Therefore, this one positive detection in an *E. crus-galli* plant after 4 weeks in the *S. jasminoides* experiment may have been caused by (cross-) contamination (in the field, or in the lab).

2.3.4 Viroid detection in six weed species after mechanical inoculation with carborundum

TASVd was not detected in any of the six mechanically inoculated weed species, while all inoculated control plants of potato, eggplant and pepper were found infected (Table 2.5). No differences were observed between both inoculum sources, i.e. *S. jasminoides* and *S. lycopersicum*.

Table 2.5 Overview of the results of the mechanical inoculation experiment. Family, Latin and common name of the inoculated plants and the number of positive plants on the total number of inoculated plants ("Positive/inoculated") are presented for both weeds species and technical controls/indicator plants.

Family	Latin name	Common name	Positive/inoculated
Asteraceae	*Chamomilla recutita*	Chamomile	0/10
	Galinsoga parviflora	Potato weed	0/10
	Sonchus oleraceus	Common sowthistle	0/10
Caryophyllaceae	*Stellaria media*	Common chickweed	0/10
Chenopodiaceae	*Chenopodium album*	Common Lamb's-quarters	0/10
Polygonaceae	*Rumex crispus*	Curly dock	0/10
Solanaceae	*Capsicum annuum*	Chillipepper	2/2
	Solanum melongena	Eggplant	2/2
	Solanum tuberosum	Potato	2/2

2.4 DISCUSSION

Three experimental approaches were used to identify potential weed hosts for viroids. First, samples of spontaneously grown weeds were taken during a survey of two commercial greenhouses in Belgium. Second, a contact experiment with infected *S. lycopersicum* and *S. jasminoides* under semi-natural conditions was used to screen spontaneously growing weeds for viroid presence. Last, six potential weed hosts were mechanically inoculated using carborundum and infected plant material and tested after six weeks.

Three species (*S. oleraceus*, *S. recta* and *B. pendula*) which had grown spontaneously in potting soils in the vicinity of PSTVd-infected *S. jasminoides* plants in an experimental growing chamber in 2010 had tested positive. These plants were in close leaf and root contact with each other. However, it is assumed that these weeds became infected via the leaves of *S. jasminoides*, because root transmission of pospiviroids has not yet been demonstrated (Antignus *et al.* 2007, Barba *et al.* 2007). While this observation prompted further investigation of weeds in pots of infected ornamentals during a survey in commercial greenhouses, no other viroid infections were found in the 8 weed samples that were analyzed. In the two greenhouses where this survey took place, several measures were taken by the growers to prevent weed growth: the use of sterilized potting soil, manual removal of weeds and the application of herbicides. In the experimental growth chamber, no weed control was applied. Additionally, the PSTVd-isolate found on the *S. jasminoides* and infected weeds in 2010 may have had a different host range and sensitivity than the TASVd isolate that was detected during the more recent survey. Furthermore, different weed species were sampled during the recent survey compared to the weed species from 2010.

During the contact experiment in the experimental greenhouse, only *E. crus-galli* tested positive for the two independent leaf samples that were taken after four weeks. However, it is assumed that this single occurrence, which contrasted with the many other negative *E. crus-galli* samplings, is not sufficient to designate this plant as a weed host. The importance of mechanical contamination when working with pospiviroids cannot be underestimated, as several positive results in the contact experiment corresponded to leaves that had been in direct contact with positive source plants.

Hence, the positive detection result for *E. crus-galli* after 4 weeks may be explained by external contamination of viroids on the leaves and/or (cross-)contamination during the analysis in the lab (plant preparation, RNA extraction, cDNA synthesis or PCR).

The mechanical inoculation experiments with TASVd conducted in this study did not reveal new hosts. Hence, our results are line with the study of Antignus *et al*. (2007), where none of the 19 weed species gave positive results after mechanical inoculation with TASVd. The mechanical inoculations performed by Antignus *et al*. (2007) were considered solid, since technical control plants were successfully infected after six weeks. Using the mechanical inoculation method, no infection by TASVd was obtained in *C. recutita* in our study. However, biolistic inoculations in this plant species have previously been successful for PSTVd (Matoušek *et al.* 2007). While the different results may have been caused by using different viroid species, they may also be explained by the use of different inoculation methods. In our study, the mechanical inoculation method was selected instead of biolististic inoculation with a Gene Gun (Matoušek *et al.* 2007) in order to mimic a natural infection as much as possible. A natural infection by a pathogen typically involves three phases: “pre-entry”, “entry” and “colonization” (Scholthof 2005). The “pre-entry” and “entry” phases comprise the transport by vectors and delivery to a plant and the penetration into the plant via existing or human-induced openings, such as microwounds induced by rubbing carborundum onto the leaves (Scholthof 2005). Next, during the “colonization” phase, the pathogen (in this case, the pospiviroid) will use specific components of the plant machinery to 1) reach the location of replication (i.e. the nucleus for pospiviroids), 2) successfully replicate itself using host-specific enzymes, and 3) systemically infect the plant via long-distance transport through the vascular tissues (Flores *et al*. 2005).

Using the biolistic inoculation procedure, cDNA/RNA gets inserted directly into the plant cell and the pre-entry and entry phases of the infection proces are skipped, therefore aiding the infection substantially. An infection like this is unlikely to happen under natural conditions and may lead to the designation of “experimental hosts” (like *C. recutita*) that present no real danger under natural and/or greenhouse conditions.

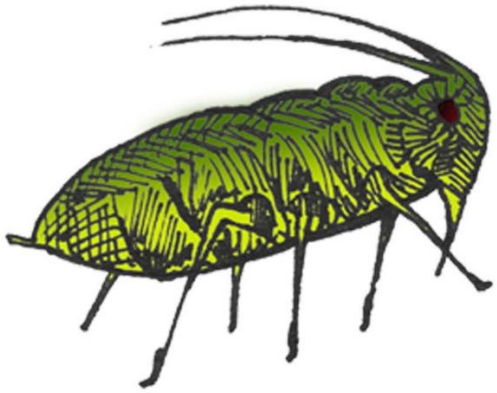
Based on the analysis of these results as well as the outcomes of previous inoculation studies, a few recommendations for future inoculation experiments are proposed. First, multiple plants of a single weed species must be inoculated and tested before its susceptibility to viroid infection can be determined. Second, every plant should be inoculated using exactly the same standard procedure and inoculum. Co-inoculation of recognized hosts as a series of technical controls is vitally important. Finally, if a weed species tests positive for viroids after several molecular detection tests, the next step is to fulfill Koch's postulates.

In conclusion, the results from the three experimental approaches used in this Chapter to investigate whether commonly occurring weeds play a role in viroid epidemiology indicate that the risk of pospiviroid transmission from weed hosts is limited. Since weeds do not appear to represent a significant phytosanitary risk as viroid reservoirs, management options for solanaceous crop cultivation should focus more on reducing the risk of pathogen entry in nations by testing and eradicating viroid-infested mother material, seeds and cuttings (EFSA Panel on Plant Health 2011, EPPO 1999) that are being traded among EU-member states.

Chapter 3:

Quantitation and localization of pospiviroids in aphids

Modified from: Van Bogaert, N., De Jonghe, K., Van Damme, E.J.M., Maes, M. and Smagghe, G. (2015). Quantitation and localization of pospiviroids in aphids. *Journal of Virological Methods*. 211, 51-54.



3.1 INTRODUCTION

Between plants, viroid movement occurs predominantly by mechanical transmission (i.e. physical contact with contaminated sources, such as infested pruning gear) (Singh 2006, Verhoeven *et al.* 2010a). Transmission routes involving different insect species, as well as more complex interactions with viruses have been proposed over the years (Antignus *et al.* 2007, Matsuura *et al.* 2010, Querci *et al.* 1997, Singh *et al.* 1999). It is conceivable that viroids are spread purely mechanically by contaminated insect body parts, surviving and causing infection when new plants are visited.

For detection and quantitation of viroids in plant tissues and seeds, several PCR-based assays have been developed over the recent years (Boonham *et al.* 2004, Botermans *et al.* 2013, Monger *et al.* 2010, Verhoeven *et al.* 2004). However, these assays have not yet been validated for insect matrices. Additionally, it is currently unknown whether insects can acquire viroids while feeding on infected plants, and if so, in which concentrations. In plants, detailed information on the ultrastructural location of viroids has been obtained by fluorescent *in situ* hybridization (FISH) in conjunction with microscopy (McFadden 1991). Detection of *Avocado sunblotch viroid* (ASBVd) in avocado leaves (Lima *et al.* 1994) and of PSTVd in reproductive organs of *Petunia* (Matsushita & Tsuda 2014) was realized using dioxygenin (DIG)-labelled RNA probes. *Coconut cadang cadang viroid* (CCCVd) and *Citrus exocortis viroid* (CEVd) have been located in plant tissues both ultrastructurally and histologically, using transmission electron microscopy (TEM) and confocal laser scanning microscopy (CLSM), respectively (Bonfiglioli *et al.* 1996).

Development of a reliable detection method for pospiviroids in insect matrixes is vital in the context of transmission research, where presence or absence of the pathogen within (or on) the insect needs to be assessed. Therefore, in this Chapter, the main objective was to localize and quantify pospiviroids in insects that had fed on pospiviroid-infected host plants. Two pospiviroids were used, namely the *Potato spindle tuber viroid* (PSTVd) and *Tomato apical stunt viroid* (TASVd). The green peach aphid *M. persicae* was chosen as a typical pest model because of two main reasons. First, they are important plant virus vectors: aphids in general account for the transmission of 50% of all insect-vectored viruses (Ng & Perry 2004).

Secondly, *M. persicae* individuals are convenient for any kind of experiment that uses feeding assays because of their polyphagous nature, which allows them to feed on a wide range of plant hosts. In the experiments of this Chapter, *M. persicae* individuals were first placed on viroid-infected plants to feed. After feeding, RNA was extracted from the aphids and used in different qPCR assays to quantify viroid copies. In parallel, FISH analyses were performed with viroid-specific probes to localize the viroids in the aphid's body by means of confocal microscopy.

3.2 MATERIAL AND METHODS

3.2.1 Insects and plants for detection and localization experiments

A colony of *M. persicae* was maintained on zucchini (*Cucurbita pepo*, L.) plants during the entire course of the experiments. Pathogen-free tomato plants (*S. lycopersicum* Cv. Marmande) and Jasmine nightshade (*S. jasminoides*) were mechanically inoculated with PSTVd (GenBank Accession N^{o}: KF49372) and TASVd (GenBank Accession N^{o}: KF484878) using carborundum, and tested viroid-positive before starting the experiments. Both the PSTVd- and TASVd-infected plants were used for the RT-qPCR detection experiments. For the localization experiments by FISH and CLSM, we made use of the TASVd-infected plants. The five PSTVd-infected plants were kept in a separate gauze cage from the five TASVd-infected plants to avoid cross-contamination.

3.2.2 Synthesis and testing of a PSTVd-transcript dilution series

3.2.2.1 Synthesis of PSTVd-transcripts

For quantitation purposes and to estimate the Limit of Detection (LOD), we made use of a standard serial dilution of pospiviroid RNA-transcripts, which were synthesized through transcription of a cloned PSTVd genome (357 nt). This sequence was inserted into the pGEM 1.2/blunt vector (Promega, Madison, WI, USA) and subsequently transformed into *E. coli* TOP10 by electroporation. Transformants were selected by ampicillin resistance. Plasmids were linearized by XbaI digestion and used as a target in an in vitro transcription reaction using the Megascript T7 kit (Thermo Fisher Scientific, Lafayette, CO, USA). This was followed by TURBO DNase digestion at 37°C for 15 min. The synthesis of the 418 nucleotide RNA (357 nt PSTVd RNA + 61 nt vector RNA) was confirmed using capillary electrophoresis (i.e. QIAxcel Advanced System, Qiagen, Hilden, Germany). RNA recovery was performed using a phenol-chloroform extraction and RNA concentration (ng/µl) was measured using a ND-1000 NanoDrop® spectrophotometer (Isogen Life Science, De Meern, the Netherlands). From this transcript, a ten-fold dilution series (10^{-1} until 10^{-10}) was prepared. To make an analogue dilution series in an aphid matrix, 2 µl of each RNA dilution was spiked onto a non-infected aphid individual. The 10 resulting aphid samples were then extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany).

3.2.2.2 Testing of the PSTVd transcript dilution series

The dilution series (pure) and the dilution series spotted on aphid individuals were tested using the one-step Agpath-ID™ RT-PCR kit (Applied Biosystems, Foster City, CA, USA) and the primers and probe of Botermans *et al.* (2013).

3.2.3 RT-qPCR detection of whole aphid individuals and dissected aphid body parts

Figure 3.1 presents a summarized pipeline of the feeding assays with aphids for RT-qPCR detection. Aphids were placed in five groups of 10 individuals into small feeding tubes that were installed upon pospiviroid-infected and non-infected plants (Figure 3.1). After an acquisition period (AP) of 24h, a subset of 23 feeding aphid individuals was selected for RT-qPCR (Figure 3.1).

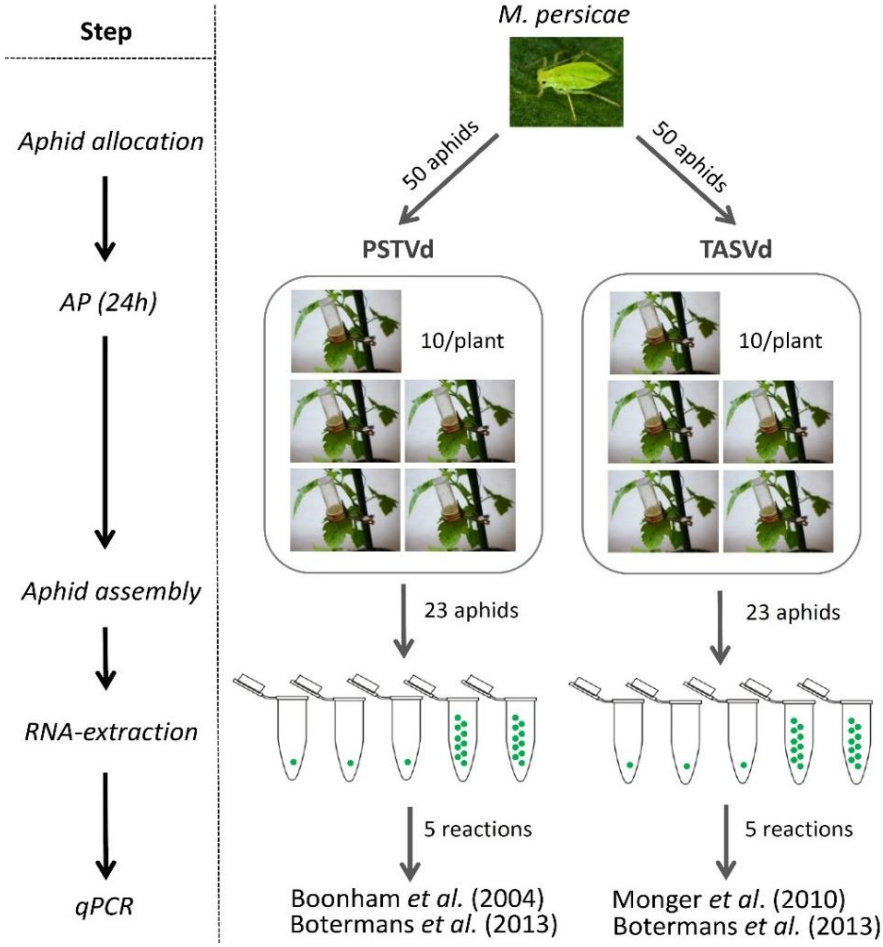


Figure 3.1: Experimental pipeline for the feeding assays with *M. persicae*. Fifty adult aphids are placed in a small feeding cage (pooled per 10) onto a leaf of a PSTVd/TASVd-infected plant. After a 24h acquisition period (AP) 23 feeding aphids are collected and divided over five reaction tubes for RNA-extraction and RT-qPCR (Boonham *et al*. 2004, Monger *et al*. 2010, Botermans *et al.* 2013).

3.2.3.1 Testing of whole aphid individuals

The sets of primers and probes used for the specific detection of PSTVd and TASVd are based on Boonham *et al.* (2004) and Monger *et al.* (2010), respectively. Next to these specific assays, generic primers and pUCCR-probe from Botermans *et al.* (2013) were used as well. For each of these qPCR assays, three separately executed tests with aphids fed on infected plants, were carried out. In each assay, five reactions containing single and pooled aphids were analyzed: three reactions containing one single aphid and two reactions containing a pooled sample of 10 aphids (Figure 3.1). Hence, during each execution of each RT-qPCR assay, 23 aphid individuals in total were analyzed.

3.2.3.2 Quantitation of viroids in aphids

In one test, conducted with the Botermans *et al.* (2013) primers and probe, the number of viroid particles present in each aphid sample was calculated. This was done by running the spiked aphid dilution series together with single and pooled aphid samples (and their technical replicates). After measuring the concentrations of the transcript dilution series using Nanodrop (see 3.2.2.1 *Synthesis of PSTVd-transcripts*), these dilutions were set as "standards" in the SDS 2.4 analysis software of the qPCR. Subsequently, the concentration (ng/µl) of the unknown aphid samples was calculated automatically by the software. Based on the average ribonucleotide molecular weight (i.e. 340 Da, Olmos *et al.* 2005), the number of copies/µl was then calculated for each of the aphid samples. The limit of detection (LOD) was defined as the lowest amount of viroid particles per aphid that can be reliably detected during a RT-qPCR assay.

3.2.3.3 Testing of dissected aphid parts

Dissected stylets, guts and embryos of in total 20 aphid individuals that were fed with PSTVd-infested tomato plants were pooled per 10 in Eppendorf tubes (i.e. two tubes of 10 guts, two tubes of 10 stylets and two tubes of 10 embryos). To prevent cross-contamination, the dissection was performed using new BD Microlance needles (25Gx5/8, Beckton Dickinson and Co, Franklin Lakes, NJ, USA) for each body part of a single aphid. Each dissected part was immediately separated from the other body parts by transferring it to an isolated drop of PBS.

These samples were tested in duplo in a one-step qPCR assay (Boonham *et al.* 2004, Agpath-ID™ one-step RT-PCR kit, Applied Biosystems) in order to confirm viroid presence/absence in these body parts. PCR cycling conditions and quantitation using the PSTVd-dilution series in aphid matrix were identical to the experiments described for whole aphid bodies (see above and Table 3.1).

3.2.4 Viroid localization using FISH

Figure 3.2 presents a summarized pipeline of the preparation of FISH samples for confocal microscopy.

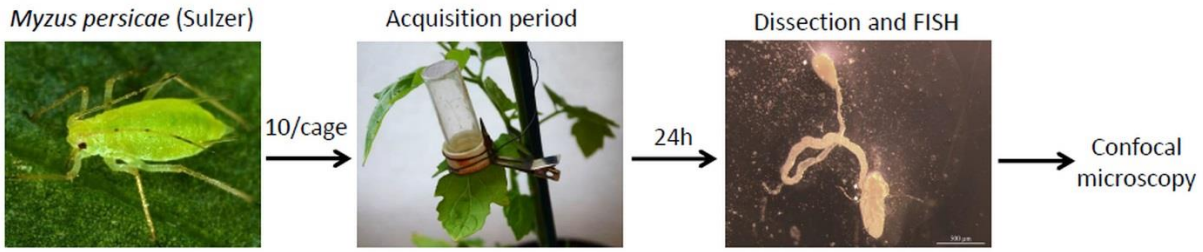


Figure 3.2: Experimental pipeline for the feeding assays with *M.persicae* and FISH localization. Ten adult aphids were placed in a small feeding cage on an TASVd-infected plant. After 24h acquisition period insects were collected and guts were dissected under a stereomicroscope. After FISH the samples were studied under a confocal microscope.

3.2.4.1 Dissection and fixation of the digestive system

For viroid localization by FISH, adult apterous aphids that had been feeding on viroid-infected plants were collected and individually dissected. The aphid's digestive system, stylet and embryos were dissected in phosphate buffered saline (PBS, pH 7.4) by means of two BD Microlance needles (25Gx5/8, Beckton Dickinson and Co, Franklin Lakes, NJ, USA) and fixed in Carnoy's fixative (chloroform:ethanol:glacial acetic acid, 6:3:1, v/v) for 5 min at room temperature.

3.2.4.2 FISH-procedure

Samples were then washed 3 times for 1 min in hybridization buffer (HB) [20mM Tris-HCL pH 8.0, 0.9M NaCl, 0.01% (w/v) sodium dodecyl sulfate (SDS), 30% (v/v) formamide], and hybridized overnight with 10 pmol fluorescent probe/ml in HB (Ghanim *et al.* 2009). Far-red Cyanine-5 (Cy5) was selected as the fluorochrome conjugated to a short oligonucleotide probe, based on an existing pospiviroid qPCR-probe (Cy5-5'-CCGGGGAAACCQGGA-3', Botermans *et al.* 2013). After hybridization, the samples were washed 3 times in HB for 1 min, and then whole-mounted and viewed under a confocal microscope.

3.2.4.3 Confocal microscopy

We used the NIS Advanced Research (AR) 4.13 software connected to a Nikon A1R confocal microscope (Nikon Instruments, Paris, France) and made use of two excitation lasers, exciting at 488 nm and 639 nm for detecting autofluorescence and the Cy5-signal, respectively. The acquisition settings and scanning settings were kept fixed throughout all experiments (i.e. scan size 512, scan speed ¼ and count 4). ROI statistics were analysed for the Cy5-channel of each picture.

3.2.5 Statistical analysis

Our dataset was analyzed in R-Studio (version 0.99.902) and STATISTICA (version 12) software (Statsoft, Tulsa, Oklahoma) using exploratory statistics and the non-parametric Kruskal-Wallis test.

3.3 RESULTS

3.3.1 Detection of viroids in aphids using three different RT-qPCR assays

Cq-values of single and pooled aphid individuals that had fed on TASVd and PSTVd infected hosts were determined during three executions of three types of RT-qPCR assays (Monger *et al.* 2010 - Table 3.1, Boonham *et al.* 2004 - Table 3.2, Botermans *et al.* 2013 - Table 3.3).

Table 3.1 Test results (Cq-value) for single (A, B, C) and pooled (D, E) aphids for three executions (Test #) of the Monger *et al.* (2010) RT-qPCR assay for aphids that had fed on TASVd-infected plants. Based on these Cq-results, the number (N°) of positive aphid samples on the total number (N°) of aphid samples are presented. ND = no detection result.

Assay	Test #	Cq-value	N° positive/total N°
Monger *et al.* 2010	1	A. 30.0 B. ND C. ND D. ND E. ND	1/5
	2	A. ND B. ND C. ND D. ND E. ND	0/5
	3	A. 28.0 B. ND C. ND D. 27.50 E. 26.40	3/5

Table 3.2 Test results (Cq-value) for single (A, B, C) and pooled (D, E) aphids for three executions (Test #) of the Boonham *et al.* (2010) RT-qPCR assay for for aphids that had fed on PSTVd-infected plants. Based on these Cq-results, the number (N°) of positive aphid samples on the total number (N°) of aphid samples are presented. ND = no detection result.

Assay	Test #	Cq-value	No positive/total No
Boonham *et al.* 2004	1	A. ND B. ND C. ND D. 32.1 E. ND	1/5
	2	A. 33.4 B. ND C. ND D. ND E. ND	1/5
	3	A. 33.0 B. ND C. ND D. 30.5 E. ND	2/5

The average Cq-value for all aphid samples (individual and pooled) over all executions of the three assays was 31 ± 3. The Cq-value of single and pooled aphid samples did not seem to be influenced by the number of aphid individuals per tube, since 1 single aphid could yield a similar, or higher, Cq-value compared to a pool of 10 (Table 3.1-3). To estimate the incidence of viroids in aphids, the percentage of positive aphid samples on the total amount of aphid samples (i.e. 5 for each execution and assay, Figure 3.2) was calculated (Table 3.1-3). This percentage was 29% on average for the three assays (Table 3.1-3).

Table 3.3: Test results (Cq-value)for single (A, B, C) and pooled (D, E per 10x) aphids for three executions (Test #) of the Botermans *et al.* (2013) RT-qPCR assay for aphids that had fed on TASVd-infected plants (*) and PSTVd-infected plants (**). Based on these Cq-results, the number (N°) of positive aphid samples on the total number (N°) of aphid samples are presented. ND = no detection result.

Assay	Test #	Cq-value	No positive/total No
Botermans *et al*. 2013	1*	A. 33.0 B. ND C. ND D. ND E. ND	1/5
	2**	A. 35.0 B. ND C. ND D. 32.5 E. ND	2/5
	3**	A. 33.8 B. ND C. ND D. ND E. 34	2/5

3.3.2 Quantification of viroids and estimation of LOD

The calculated numbers of transcripts for 1 single individual and 1 pooled sample of aphids (i.e. 10 individuals) after conducting an analytical sensitivity assay using the Botermans-primers (one-step RT-qPCR) are presented in Table 3.4. Based on these data, the limit of detection (LOD) was estimated at $1.69*10^6$ copies for one whole aphid body in this specific test. The RT-qPCR efficiency of the standard dilution series was 91% (calculated by equation: *efficiency* = $-1+10^{(-1/slope)}$, Table 3.4), making this series suitable for relative viroid quantitation in aphid individuals.

Table 3.4 RT-qPCR analysis of PSTVd transcripts in *M. persicae* aphids. Mean Cq values ± Standard Deviation (SD) are presented for a test sample and its technical replicate. Calculated concentrations of viroid RNA (ng/µl) and number of viroid copies (per µl) for the test sample are shown. The PSTVd - transcript standard curve has a R^2 of 0.998 and a slope of 3.56.

Sample contents	Mean Cq ± SD	Concentration (ng/µl)	Number of copies (per µl)
1x *M. persicae*	30.57 ± 0.17	0.00398	$1.69*10^7$
10x *M. persicae*	23.64 ± 0.61	0.352	$1.49*10^9$
PSTVd-transcript 10^{-2}	19.29 ± 0.45	3.96	$1.68*10^{10}$
PSTVd-transcript 10^{-3}	24.46 ± 0.20	0.396	$1.68*10^9$
PSTVd-transcript 10^{-4}	26.77 ± 0.34	0.0396	$1.68*10^8$
PSTVd-transcript 10^{-5}	30.54 ± 0.50	0.00396	$1.68*10^7$
PSTVd-transcript 10^{-6}	34.03 ± 0.70	0.000396	$1.68*10^6$

3.3.3 Localization results

For the localization experiments using FISH and CLSM, the specificity of detection was confirmed using the appropriate controls (Table 3.5). A clear Cy5 fluorescent signal was observed in the foregut of a probe-treated aphid that had fed on a TASVd-infected plant (Figure 3.3 A-B). Limited background autofluorescence was observed in the gut of aphids that did not feed on a viroid-infected plant, and/or not hybridized with the fluorescent probe (Figure 3.3 C-E). A visual distinction between a positive signal and autofluorescence could not be made for the stylets because of high autofluorescence (Figure 3.4 A-B). No viroid-related signals were observed in the embryos of the aphid bodies (Figure 3.4 C-D).

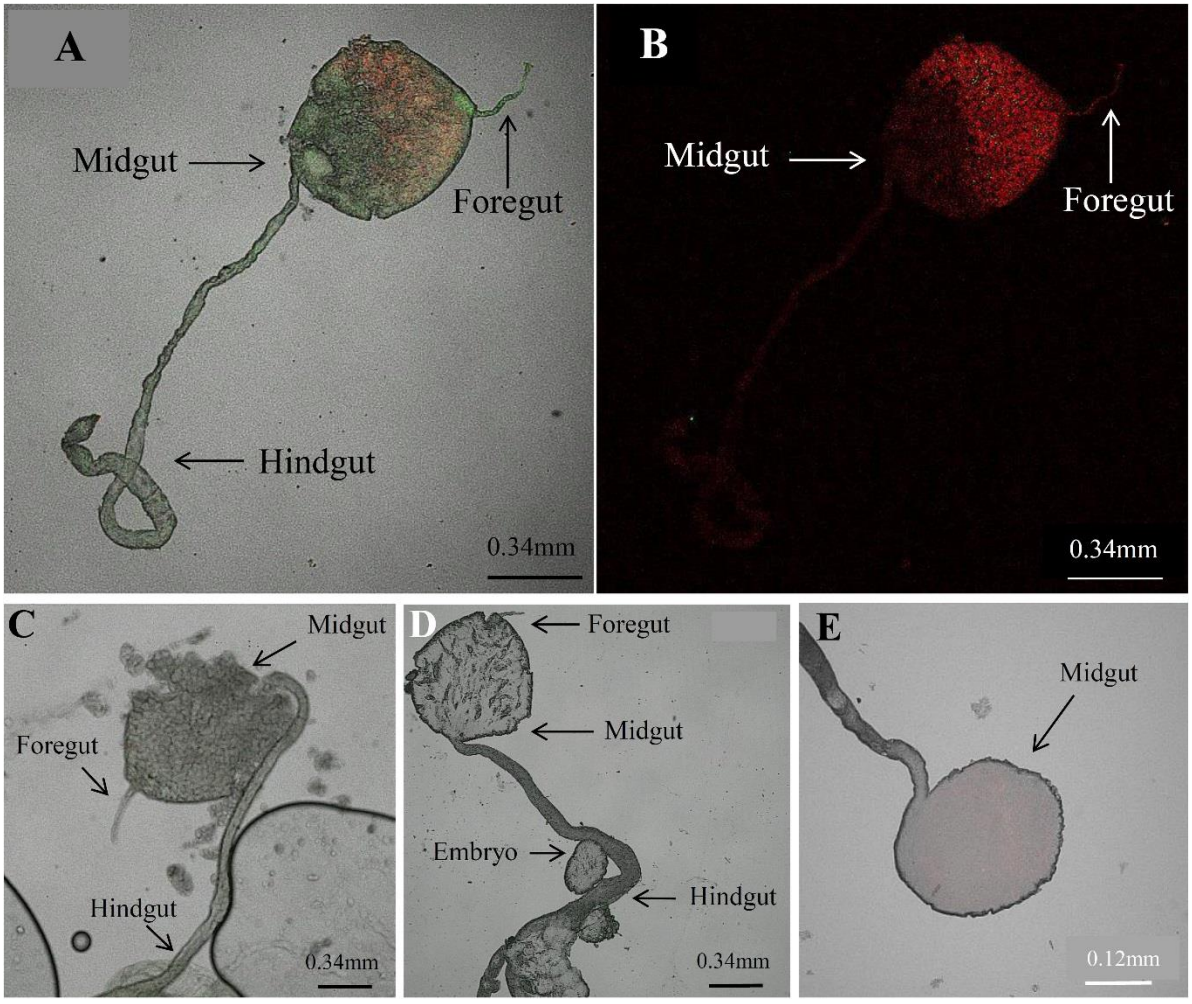


Figure 3.3: Confocal laser-scanning microscopy of the green peach aphid (*M. persicae*). The gut of an aphid after feeding for 24h on TASVd-infected plants is presented (A-B). A: a composite picture with fluorescence from all channels, B: only Cy5- fluorescence for the same specimen. C-E: Composite pictures with fluorescence from all channels of negative controls showing guts of aphids that did not feed on infected material.

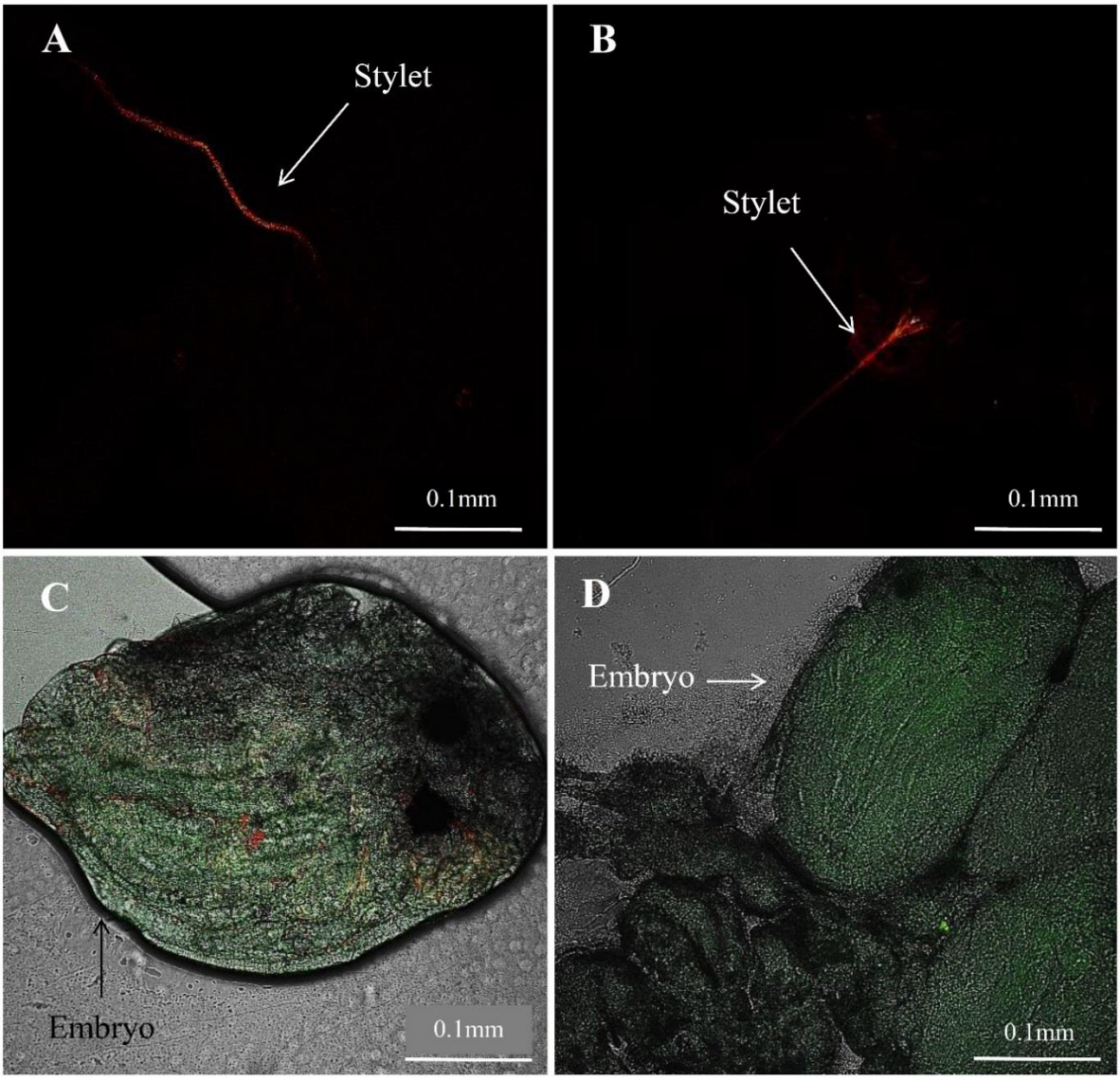


Figure 3.4: Confocal laser-scanning microscopy of the green peach aphid (*M. persicae*). A-C: Dissected stylets and embryos of aphids fed on TASVd-infected material are presented next to negative controls of the same body parts (B-D).

To assess potential differences between the four treatments of aphids (i.e. fed on infected/non-infected plants, treated with/without FISH-probe) fluorescence intensities of a total of 62 aphid guts over four treatment groups (Table 3.5) were analyzed. Statistical analysis shows that the "positive probe" treatment had a significantly higher fluorescence intensity when compared to the other three groups ($p=0.0022$, Kruskal-Wallis). The fluorescence intensities were considerably higher in aphids fed with TASVd-infected material and hybridized with Cy5-probe, which proves that the applied technique enables to visualize presence of the viroid in the aphid organs.

Table 3.5 FISH analysis of viroids in the gut of green peach aphid. Mean fluorescence intensities were calculated for a total of 62 aphid guts over four treatment groups (image size 286.41x286.41 microns). The "Positive, + probe" treatment consists of aphids fed on infected leaves and treated with probe, in contrast to the other three negative control treatments (i.e. "Negative, - probe", "Negative, + probe" and "Positive, - probe). [1]Values followed by the same letter are not significantly different at α = 0.05.

Test design	Outcome	Mean fluorescence intensity ± SD[1]
Negative, - probe	Autofluorescence	71.2 ± 59.1*a*
Negative, + probe	Autofluorescence	100.5 ± 57.3*a*
Positive, - probe	Autofluorescence	89.1 ± 53.6*a*
Positive, + probe	Cy5 and autofluorescence	227.9 ± 164.5*b*

3.3.4 Testing of dissected body parts

The qPCR-assays conducted on two pools of 10 dissected guts and embryos of aphids fed on positive plant material, confirmed the localization results obtained via FISH. The pools of 10 dissected guts resulted in a mean Cq of 31.81 ± 1.5. Pooled embryos, however, did not give a positive Cq-value, confirming the absence of Cy5-signal in embryos during microscopy. The mean Cq for 10 dissected stylets was 30.67 ± 2.6. Experiments containing only 1 dissected stylet, gut or embryo did not give positive results, probably because of concentrations under the detection limit (data not shown).

3.4 DISCUSSION

In this Chapter, qPCR and FISH experiments were conducted in order to detect and localize pospiviroids in aphids that had fed on infected hosts. The FISH experiments revealed the presence of viroids in the stylet and foregut of the aphid, but not in the rest of the body. It is therefore hypothesized that vertical transmission of viroids through *M. persicae* will probably not take place, since signals were absent in the embryos.

In the qPCR-experiments, it was observed that not all aphids that had fed on infected plants yielded positive results. This might be partially due to the fact that samples were below the detection limit and/or that potential contaminants in the aphid extracts might inhibit the efficiency of the qPCR reaction. Additionally, our result lies in the same range of previous prevalences calculated for non-persistently transmitted viruses (Moreno *et al.* 2009, Olmos *et al*. 2009). For instance, for the non-persistently transmitted *Plum Pox Virus* (PPV), the percentage of positive amplifications after RT-qPCR in individual *M. persicae* individuals was 22% after different APs ranging between 5 minutes and 2 hours (Olmos *et al.* 2005) and 13.6% after an AP of 5 to 10 minutes (Moreno *et al*. 2009). Consequently, only part of the total number of aphids submitted experimentally to different feeding periods acquires detectable levels of RNA-PPV (Olmos *et al.* 2005). Olmos *et al.* (2005) concluded that not all the aphids that are feeding on infected plant material effectively sting into plant cells that are containing virions.

Biological variations, like the varying concentrations of virus in the leaves, as well as the length of feeding time on infested material and/or the specific time point at which individuals were selected for analysis, may influence the prevalence of viruses detected in aphids (Moreno *et al*. 2009, Olmos *et al.* 2005, Tamada & Harrison 1981). Additionally, several studies have pointed out that the number of cycles necessary for RT-qPCR detection of viral targets in single aphids is usually higher than in plant samples (Fabre *et al.* 2003, Moreno *et al*. 2009, Olmos *et al.* 2005, Saponari *et al*. 2008). For non-persistent viruses, this observation can be explained by the fact that these viruses do not replicate in the aphid body, leading to a low number of viral copies detected in an individual aphid (= high Cq values) (Moreno *et al*. 2009). In case of pospiviroid acquisition and transmission, more detailed experiments with time as a varying factor could yield further insights.

It must be noted that the results of the RT-qPCRs that were performed in our study to calculate the prevalence of positive aphid samples using three different assays (Boonham *et al*. 2004, Botermans *et al.* 2013, Monger *et al*. 2010*)* can only be interpreted in a semi-quantitative way since reaction efficiencies are not provided. An accurate quantification of the number of PSTVd transcripts in aphids was only done once using the Botermans *et al*. (2013) primers and resulted in a relative qRT-PCR efficiency of 91% and $1.7*10^6$ as the lowest amount of copies that could still be reliably detected (= LOD). Before the start of the experiments described in this Chapter, all three qPCR assays (Boonham *et al*. 2004, Botermans *et al.* 2013, Monger *et al*. 2010*)* were validated according to the EPPO standards for accredited plant pest diagnostic labs (EPPO 2014) using viroid samples and transcripts from the collection of the Diagnostic Center for Plants (DCP, ILVO). Apart from viroid test samples, internal controls (Cox/Nad5 primers), technical replicates and negative, positive and "no template" controls (ntc) were co-analyzed during these validations. Furthermore, qPCRs with low efficiencies or abnormalities were repeated. These validations resulted in a Cq of 35 as cut-off value (see Table 2.2, Chapter 2). Furthermore, during the synthesis of an RNA transcript dilution series, critical points required to generate trustworthy absolute standards were acknowledged: e.g. the standard RNA is a single pure species, pippeting was done accurately during the synthesis of the dilutions and small aliquots of the dilution series were stored at -80°C and thawed only before use in order to assure their stability (Applied Biosystems, 2010).

In summary, the results of this Chapter show that viroids can be detected and localized in aphids using a combination of FISH and qPCR. While fluorescence intensities and Cq values are relatively low, the presence of viroid particles in aphid material cannot be denied after evaluating negative control pictures and ROI statistics. The two main advantages of working with short oligonucleotide probes, as used in this study, are the swift penetration through tissues and the short processing time of the samples, allowing a rapid detection (Ghanim *et al.* 2009). With the acquired quantitation and localization technologies, the next step in this research is to address the biological question whether viroid transmission can occur. It is hypothesized that viroids could be transmitted by means of mechanical contact through contaminated mouth and body parts of insect vectors like aphids, but also by feeding, since presence of viroids in the insect (aphid) gut has clearly been demonstrated in this study.

Chapter 4:

Assessment of pospiviroid transmission by *Myzus persicae*, *Macrolophus pygmaeus* and *Bombus terrestris*

Modified from: Van Bogaert, N., Olivier, T., Bragard, C., Maes, M., Smagghe, G., and De Jonghe, K. (2016). Assessment of pospiviroid transmission by *Myzus persicae*, *Macrolophus pygmaeus* and *Bombus terrestris*. *European Journal of Plant Pathology*, 144(2), 289-296.

4.1 INTRODUCTION

During the past decade, pospiviroid outbreaks in tomato and sweet pepper crops have been regularly reported worldwide. Despite demonstration of infected seeds and latently infected ornamentals leading to outbreaks (Navarro *et al*. 2009, Parella & Numitone 2014, Shiraishi *et al.* 2013, Van Brunschot *et al.* 2014, Verhoeven *et al*. 2010d & 2012), transmission due to insect vectors or mechanical contact cannot be excluded. This hypothesis is supported by several elements: the phylogenetic linkage between tomato and ornamental pospiviroid isolates, the unsuccessful upstream tracing of the agents in some outbreaks (Verhoeven *et al.*, 2004), the prevalence of pospiviroids in wild and ornamental plant species (EFSA Panel on Plant Health 2011, Barbetti *et al.* 2012) and the demonstrated vectoring capacity of certain insects (Antignus *et al.* 2007, De Bokx & Piron 1981, Matsuura *et al.* 2010, Schuman *et al.* 1980).

Recently, the European Food and Safety Authority (EFSA) estimated the probability of pospiviroid transmission by aphids or bumblebees, within and between crops, as an "unlikely to moderately likely event" (EFSA Panel on Plant Health, 2011). At the same time, EFSA also stated that this assessment is uncertain due to limited experimental data (EFSA Panel on Plant Health, 2011). Additionally, data on insect transmission of viroids contain many conflicting results, probably because of inaccurate assays, diagnostic problems and mechanical contamination. The latest viroid-insect transmission study by Nielsen *et al.* (2012) recorded no viroid transmission via insects, therefore not confirming bumblebee-mediated transmission of viroids that was shown in earlier studies (Antignus *et al.* 2007, Matsuura *et al.* 2010).

In this Chapter, the main goal was to investigate transmission of pospiviroids by three insects, each belonging to a different functional group (i.e. a pest species, a pollinating insect and a polyphagous biological control agent) but all typically occurring in viroid-suseptible solanaceous species. The green peach aphid *M. persicae* was selected for its widespread and reccurent presence in susceptible crops and its well-established role as plant virus vector. Pollinators were represented by the buff-tailed bumblebee *B. terrestris*. The foraging activity of bumblebees has been implicated in plant virus transmission before (Li *et al.* 2014, Shipp *et al.* 2008).

Lacasa *et al*. (2003) showed that bumblebees could acquire *Pepino mosaic virus* (PepMV) on their legs by way of infected pollen and extracts of their legs with adhering pollen could infect healthy tomato plants. In the case of viroids, this type of contact between infected pollen and/or contaminated bodyparts and the plant could lead to both intra-and interspecies transmission. The third insect, the polyphagous bug species *M. pygmaeus*, was selected because of its increasing popularity as a biological control agent in the commercial cultivation of many crops. Additionally, *M. pygmaeus* is a potential candidate for pollen-mediated transmission, since it can complete its life cycle by feeding on pollen (Vandekerkhove & De Clercq 2010). Furthermore, many viroids are detected in pollen of infected plants (Barba *et al.* 2007, Singh *et al.* 1992b, Zhu *et al.* 2002) and pollen-mediated transmission has already been shown for several viroids (Barba *et al.* 2007, Kryczynski *et al.* 1988). For each of these three insects, various transmission experiments were organized using different pospiviroid isolates and host plants in order to assess different combinations of plants (inter-and intraspecific transmission) and viroids.

4.2 MATERIAL AND METHODS

4.2.1 Experimental set-up

To evaluate whether *M. persicae*, *M. pygmaeus* and *B. terrestris* could function as vectors for pospiviroids, four transmission experiments were organized for each insect (Table 4.1). During these experiments, insects were placed in cages together with different species of pospiviroid-infected and non-infected host plants. The experiments differed in terms of number and species of host plants, pospiviroid inoculum and ambient temperature of the experiment. The pospiviroid isolates used in this study were: *Potato spindle tuber viroid* (PSTVd), *Tomato apical stunt viroid* (TASVd), *Tomato chlorotic dwarf viroid* (TCDVd) and *Pepper chat fruit viroid* (PCFVd) (Table 4.1). The original PCFVd-isolate was kindly provided by Dr. Verhoeven (NPPO, the Netherlands). The different host plants were tomato (*S. lycopersicum*), chili pepper (*Capsicum chinense*, L.), petunia (*Petunia x hybrida*) and tobacco (*Nicotiana benthamiana* L.) (Table 4.1). Infected source plants were inoculated at a young stage (4-6 leaves) using infected plant sap that was applied on carborundum-dusted leaves (Verhoeven & Roenhorst, 2000).

Table 4.1: Overview of the transmission experiments conducted for bumblebees (*B. terrestris*), green peach aphids (*M. persicae*) and whitefly predatory bugs (*M. pygmaeus*r) in the period 2012-2015. Column headings: Exp. N^0 = experiment number and location: * = CRA-W, Gembloux, Belgium or ** = ILVO, Merelbeke, Belgium, Viroid isolate = inoculated viroid isolate and GenBank Accession N^0, AT = ambient temperature (°C).

Insect species	Exp. N^0	Pospiviroid	N^0 of insects	AT (°C)	Source plants (SP)	N^0 SP	Receiving plants (RP)	N^0 RP
M. persicae	1**	TASVd (KF484879)	100	23	*S. lycopersicum* cv. Marmande	1	*S. lycopersicum* cv. Marmande	3
	2**	TASVd (KF484879)	10	20	*Physalis* sp.	1	*Physalis* sp.	1
	3**	TASVd (KF484879)	10	20	*N. benthamiana*	1	*N. benthamiana*	1
	4**	TASVd (KF484879)	50	20	*C. chinense* cv. Mme Jeanette	1	*C. chinense* cv. Mme Jeanette	3
M. pygmaeus	5**	TASVd (KF484879)	100	23	*S. lycopersicum* cv. Marmande	1	*S. lycopersicum* cv. Marmande	3
	6**	PSTVd (FM998542)	100	23	*S. lycopersicum* cv. Marmande	1	*S. lycopersicum* cv. Marmande	1
	7**	TASVd (KF484879)	50	20	pollen of *Petunia x hybrida*	1g	*S. lycopersicum* cv. Marmande	1
	8**	PCFVd (FJ409044)	100	23	*C. chinense* cv. Mme Jeanette	1	*C. chinense* cv. Mme Jeanette	3
B. terrestris	9*	TCDVd (HG739070)	50	19	*Petunia x hybrida*	33	*S. lycopersicum* cv. Minibel	18
	10*	TCDVd (HG739070)	50	25	*Petunia x hybrida*	16	*S. lycopersicum* cv. Minibel	18
	11**	TASVd (KF484879)	50	20	*S. lycopersicum* cv. Marmande and *N. benthamiana*	12	*S. lycopersicum* cv. Marmande	3
	12**	TASVd (KF484879)	50	23	*S. lycopersicum* cv. Marmande and *Petunia x hybrida*	2	*Petunia x hybrida*	8

4.2.2 Experiments with *M. persicae* and *M. pygmaeus*

A colony of *M. persicae* individuals was maintained on zucchini, and a colony of *M. pygmaeus* was maintained on viroid-free tomato seedlings and pollen (Biobest, Westerlo, Belgium) throughout the course of the experiments. At the start of each experiment, adult insects of *M. persicae* and *M. pygmaeus* (Exp. 1-8, Table 4.1) were placed onto leaves of pospiviroid-infected host plants in a medium-sized gauze cage (60 x 60 x 90 cm, mesh size = 0.8 x 0.8 mm) in a climate chamber (3 x 2 m). After an acquisition period of two days, healthy host plants were placed inside the cage, ensuring that the distance between healthy and infected plants was large enough to avoid any contact (± 30 cm).

To investigate pollen-mediated transmission of viroids by *M. pygmaeus*, 50 adults were fed with 1g of pollen from TASVd-infected *Petunia x hybrida* plants, of which 100 mg had been tested positive using the pospiI-FW/RE primers (Verhoeven *et al.*, 2004) using identical reagents as for plant testing described below. The insects were allowed to feed on this pollen during two days in a gauze cage (30 x 30 x 30 cm, mesh size = 0.8 x 0.8 mm). After this period, the individuals were placed onto healthy flowering tomato plants, which were tested after six weeks.

4.2.3 Experiments with *B. terrestris*

For *B. terrestris* (Exp. 9-12, Table 4.1), a Mini-Hive (Biobest, Westerlo, Belgium) containing approximately 50 adult bumblebees was placed at a distance of 1 m from the pospiviroid-infected host plants (e.g. Figure 4.2). Bumblebee experiments 9 and 10 (Table 4.1) were conducted in a greenhouse compartment (2.65 x 2.3 m) at CRA-W (Walloon Agricultural Research Centre, Gembloux). The other two bumblebee experiments (Exp. 11-12 , Table 4.1) were carried out in a gauze cage (1.75 x 1.75 x 1.75 m, mesh size = 0.8 x 0.8 mm) placed inside a greenhouse compartment (4.7 x 4.7 m) at ILVO (Institute for Agricultural and Fisheries Research, Merelbeke). During all experiments, insect activity (i.e. flying, foraging) was closely monitored via visual observations throughout six weeks. After these six weeks a first plant sampling and PCR-testing was conducted.

4.2.4 Plant sampling and testing

After 6 weeks a random leaf, flower and/or fruit (if these had been formed) sample was analyzed for each source (i.e. infected) and receiving (i.e initially non-infected) plant in the experiments with *M. pygmaeus* and *M. persicae* (Exp. 1-8, Table 4.1). For the bumblebee experiments, a random leaf, flower and/or fruit (if present) sample was taken after 4, 6, 8 and 12 weeks (Exp. 9-12). After crushing, total RNA was extracted from 100 mg of plant material using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA) and cDNA was synthesized using the iScript^{TM} cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). For the generic detection of pospiviroids, the pospi1-FW/RE primers (Verhoeven *et al.* 2004) and the pospi1deg-FW and pospi1s-RE (Olivier *et al.* 2014) were used.

After agarose gel-electrophoresis, amplicons of the expected size were isolated and sent for sequencing (Macrogen Europe, Amsterdam, the Netherlands). When weak or ambiguous signals were detected during electrophoresis, the viroid RNA content of samples was re-analysed using an RT-qPCR with the Agpath-ID^{TM} one-step RT qPCR Kit (Applied Biosystems, Foster City, CA,USA) using the primers and probes of Botermans *et al.* (2013), Boonham *et al.* (2004) and Monger *et al.* (2010). All necessary diagnostic controls were taken into account during the analyses (i.e. a healthy tomato and a no template control). Based on validation data, Cq-values higher than 35 were considered to be negative (see Chapter 2, Table 2.2).

4.2.5 Insect sampling and testing

After an acquisition period of two days, five *M. persicae* and five *M. pygmaeus* individuals that had been observed probing during each of the four experiments organized per species (Table 4.1), were individually crushed in liquid nitrogen using 2 ml microtubes (Exp. 1-8, Table 4.1). RNA was subsequently extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany). Additionally, 15 and 10 *B. terrestris* individuals that were actively foraging on flowers of infected plants were captured during experiments 11 and 12 (Table 4.1). To avoid excess material for RNA-extraction, bumblebee body parts were first dissected in head, thorax plus abdomen and legs, using sterile micro-scissors (Vannas scissors No.14003, World Precision Instruments, Sarasota, FL, USA). The micro-scissors were decontaminated using 1% Virkon and rinsed three times with distilled water, first during the dissection of the different body parts of an individual, and then between each individual dissection.

The legs of a bumblebee individual were pooled together in one reaction and thorax and abdomen of one individual were analyzed together in one reaction. A volume of 1.5 ml RLT buffer RNeasy Mini kit (Qiagen, Hilden, Germany) was used to start the RNA-extraction. After centrifuging this mixture at 14.000 rpm, 500 µl of the supernatans was used to continue the extraction procedure according to the manufacturer's conditions. Pospiviroid detection was done via RT-qPCR as described above.

Sensitivity of detection of a PSTVd-dilution series in *M. pygmaeus* and *B. terrestris* matrices was first evaluated in a separate test (see 4.3 Results, Table 4.1). This test was based on a similar detection test done with the same PSTVd-dilution series and *M. persicae* aphids of Chapter 3. After pipetting 1 µl of each dilution onto a *B. terrestris* individual and a *M. pygmaeus* individual per reaction, RNA was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany). Amplification was done using the primers of Boonham *et al.* (2004) and the one-step AgPath ID™ (Applied Biosystems, Foster City, CA, USA). A technical replicate was pippeted into the qPCR plate for each single sample.

4.2.6 Phytosanitary precautions

The risk of mechanical contamination due to human handling of the plants or contact with infected plants was minimized as all conceivable phytosanitary precautions were taken, including physical separation of infected and healthy plants, placing the plants in separate saucers and attaching them to supporting sticks to prevent them from leaning over. Additionally, new sterile gloves were used to sample each individual plant and care was taken to prevent diffuse spraying while watering plants.

4.3 RESULTS

4.3.1 Analytical sensitivity for the detection in *M. pygmaeus* and *B. terrestris* matrices

Table 4.2 presents the results of a one-step RT-qPCR test of a PSTVd dilution series that was spotted onto two different insect matrices: *M. pygmaeus* and *B. terrestris* individuals. Based on the results of this test, Cq-values greater than 35 were considered to be negative since the PCR efficiency decreases from this value onwards. Hence, in further qPCR tests with *M. pygmaeus* and *B. terrestris*, a Cq-value of 35 is maintained as cut-off.

Table 4.2: Mean Cq-values after conducting one-step RT-qPCR tests of a PSTVd dilution series (10^{-3}-10^{-10}) in three different matrices: 1) as pure RNA transcripts, 2) transcripts spotted on *M. pygmaeus* bodies and 3) transcripts spotted onto *B. terrestris* individuals. Mean Cq values and standard deviations were calculated for each sample and its technical replicate.

Dilution series	Cq (transcripts)	Cq (*M. pygmaeus*)	Cq (*B. terrestris*)
10^{-3}	9.7 ± 0.71	18.9 ± 0.14	18.9 ± 0.42
10^{-4}	13.5 ± 0.57	21.8 ± 0.28	20.9 ± 0.14
10^{-5}	16.8 ± 0.28	23.9 ± 1.13	24.9 ± 0.85
10^{-6}	19.8 ± 0.81	27.7 ± 0.42	29.3 ± 0.28
10^{-7}	23.4 ± 0.55	30.9 ± 0.21	33.1 ± 0.14
10^{-8}	27.1 ± 0.28	35.3 ± 0.57	33.5 ± 0.42
10^{-9}	30.4 ± 0.85	35.9 ± 2.47	35.4 ± 0.85
10^{-10}	34.3 ± 0.99	37.2 ± 1.84	35.7 ± 0.71

4.3.2 Transmission experiments

Bumblebees, aphids and whitefly predatory bugs were regularly observed feeding on infected and healthy plants during all transmission experiments.

4.3.2.1 Experiments with M. persicae and M. pygmaeus

In the case of *M. persicae*, all 20 individuals sampled over four different experiments tested positive for pospiviroids after the AP of two days.

All 20 samples of *M. pygmaeus* individuals tested negative after the AP. In the transmission experiments with *M. persicae* and *M. pygmaeus*, none of the receiving tomato plants tested positive after six weeks.

4.3.2.2 Experiments with bumblebees

One *B. terrestris* tested positive in experiment No. 11 (Table 4.1) where the legs of one individual resulted in a Cq-value of 34.7 after conducting the one-step RT-qPCR assay designed by Botermans *et al.* (2013). The 24 other bumblebee individuals tested negative. In bumblebee experiment N^0 11, two samples of (initially healthy) tomato flowers tested positive in a one-step RT-qPCR conducted after four weeks (Cq = 31.7 and Cq = 34.5; Botermans *et al.* 2013). However, when the same plants were resampled two weeks later, all samples were negative, indicating that systemic spread from the flowers to the rest of the plant had not occurred or was not detectable.

In bumblebee experiment N^0 10 (Table 4.1; Figure 4.1) the flower sample of one of the 18 initially healthy tomato plants (sampled two months after the start of the experiment) tested positive after an RT-PCR with primers of Olivier *et al.* (2014, plant N^0 17 in Figure 4.1). After four months, both leaf and fruit samples of this plant tested positive using the same PCR-test as before (Lane N^0 17, Figure 4.2 A-B). For these samples, clear bands at the expected size ± 200 nt were observed after gel-electrophoresis.

The sequence of the amplicons obtained at two and four months showed a perfect similarity with the sequence of the TCDVd isolate in the petunia source plants (GenBank Accession N^0 HG739070). None of the other tomato plants (Lane N^0 1-16 and Lane 18, Figure 4.2) tested positive. In bumblebee experiment N^0 9 (Table 4.2) none of the 18 receiving tomato plants tested positive. The slight non-specific bands in lanes 11,12 and 13 (Figure 4.2 A) were also sent for sequencing and a BLAST search was conducted, however, no matches were found. These results were also confirmed by a RT-qPCR using the appropriate primers and probe of Botermans *et al.* (2013).

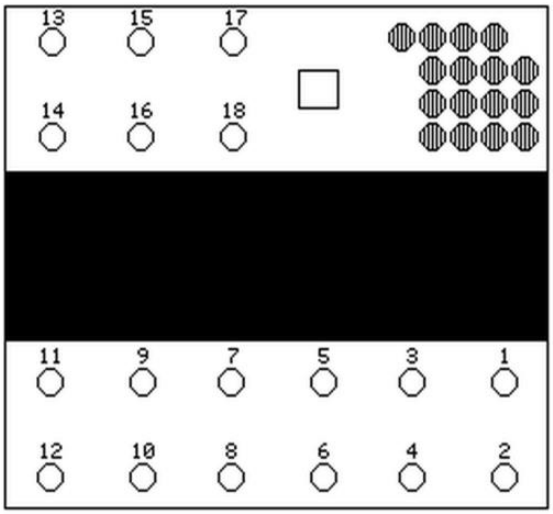


Figure 4.1: Experimental lay-out of Exp. N^0 10 with *B. terrestris* conducted in a greenhouse at CRA-W. Empty circles = 18 initially healthy tomato plants in individual saucers placed onto two (white) benches separated by a (black) corridor, empty rectangle = bumblebee hive, lined circles = 16 TCDVd-infected petunia plants. Plant N^0 17 got infected with TCDVd. Figure by Thibaut Olivier (CRA-W, Gembloux).

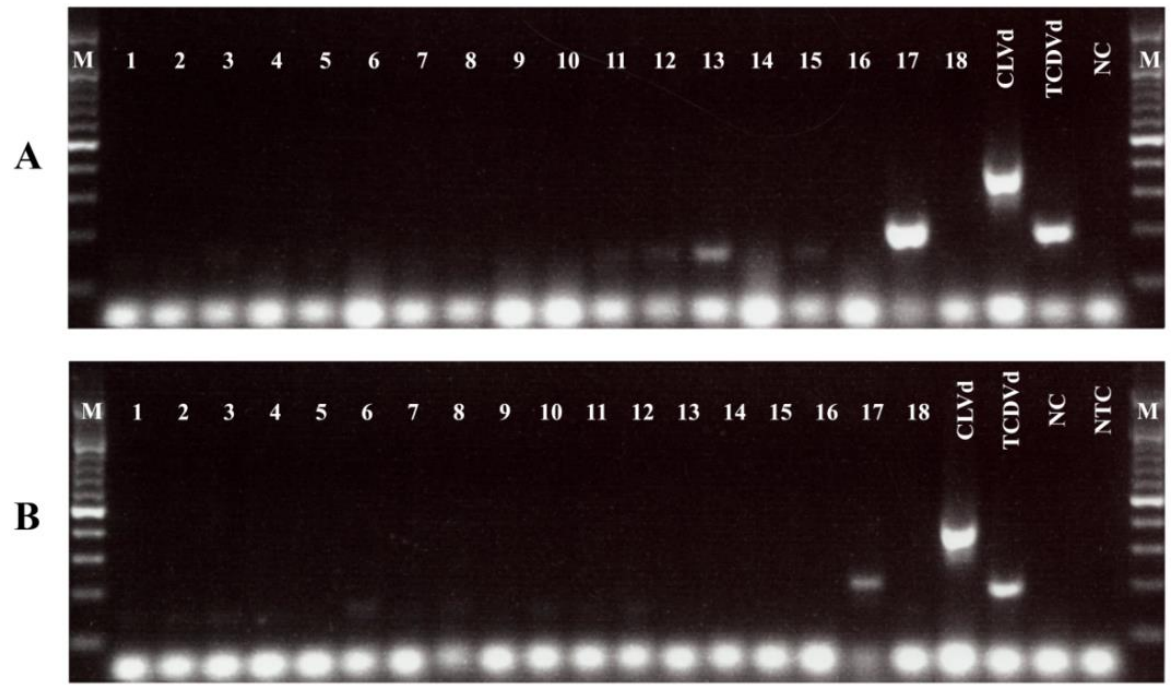


Figure 4.2: Gel pictures of A) leaf samples and B) fruit samples of 18 initially healthy tomato plants after four months of bumblebee transmission (amplicons obtained by RT-PCR, Olivier *et al.* 2014). "M" = Molecular weight markers (O' GeneRuler 100 bp Plus DNA Ladder, Thermo Fisher Scientific, Waltham, MA, USA), Lane "1-18" = initially healthy tomato plants, "CLVd" = CLVd positive control (size 375nt), "TCDVd"= TCDVd positive control (size 195 nt), NC = negative tomato control, NTC = blank control. Primer dimers (± 50 nt) are observed in all lanes and faint nonspecific bands (± 165 nt) in lanes 11, 12 and 13 of panel A. Figure by Thibaut Olivier (CRA-W, Gembloux).

4.4 DISCUSSION

Little information is available on natural pospiviroid infections in susceptible crops. However, in several cases, the primary infections appeared either at a unique location or in patches throughout the crop. These primary infections then spread along rows of the crop as the growing season progresses (Mackie *et al.* 2002, Verhoeven *et al.* 2004, Verhoeven *et al.* 2007). While the latter spreading can be attributed to mechanical transmission during pruning or staking, the scattered pattern of primary infections may suggest a low-level transmission via seeds or insects.

The present study on insect transmission revealed that no viroids were transmitted by *M. persicae*, although all twenty sampled individuals had tested positive. It is remarkable that all tested aphid individuals gave a positive detection result, since the prevalence of positive amplifications was much lower in previous experiments (Chapter 3); i.e. 29%. However, as discussed earlier, virus detections in single aphids that have been feeding on infected plants, are highly variable and influenced by many factors (Fabre *et al*. 2003, Moreno *et al*. 2009, Olmos *et al.* 1997, Olmos *et al.* 2005, Saponari *et al.* 2008, Tamada & Harrison 1981). The prolonged acquisition period of two days in this Chapter compared to 24h in Chapter 3 may also be crucial in this respect. Possible explanations why *M. persicae* is unable to transmit are that viroids are not retained at the extreme tip of the stylet or on the claws, as is the case for non-persistent viruses (Uzest *et al.* 2007) and for *Tobacco mosaic virus (*Bradley & Harris, 1972) respectively. Low viroid concentrations (Chapter 3), dsRNA degradation activity observed in aphid saliva (Christiaens *et al.* 2014) or the absence of an assisting plant virus in which the viroid could be transencapsidated (Querci *et al.* 1997) could also explain the lack of transmission. Regardless of the explanation, the results of the present study are in line with those previously obtained by De Bokx & Piron (1981) for *M. persicae* and suggests that pospiviroids are degraded in the foregut of the green peach aphid preventing circulative transmission. The risk posed by *M. persicae* regarding pospiviroid transmission is thus considered to be negligible.

In the transmission experiments with *M. pygmaeus*, all 20 insects tested negative despite the fact that this bug partially shares the same feeding mode (i.e. stylet penetration of the vascular tissue of the plant) with aphids.

The PCFVd transmission experiment was performed with *Capsicum chinense* as a donor plant since it was observed that *M. pygmaeus* preferred chili peppers over tomatoes.

However, the hypothesis that the attractiveness of the food source could induce a positive transmission was not confirmed here. Consumption of TASVd-infected pollen did not lead to transmission events either. Although it cannot be completely excluded that insect extracts could have some inhibitory effects on RT-PCR detection and/or that the pospiviroid concentrations were possibly below the detection limit, our results could also be explained by an early degradation of viroids due to salivary enzyme activity. This latter hypothesis is supported by the dsRNA degradation activity observed in the saliva of another member of the *Miridae* family: *Lygus lineolaris* (Palisot de Beauvois) (Nault 1997) whereas another plant bug was found to transmit PSTVd at a low rate on potato (Schumann *et al.* 1980). Considering the relatively high number of *M. pygmaeus* individuals used in the four experiments in this study as well as the absence of positive receptor plants and insects, we conclude that the importance of *M. pygmaeus* as a potential vector for pospiviroids is minimal.

In only one bumblebee experiment (N^0 10), one out of 18 initially healthy tomato plants tested positive for TCDVd. When the same plant was resampled after four months, both fruit and leaf samples tested positive and sequencing confirmed that the viroid isolate was identical to the TCDVd isolate of the 16 infected donor petunias. Therefore, we concluded that this particular tomato plant got infected as a result of bumblebee activity. In contrast, two positive detection results of tomato flowers after 4 weeks in another bumblebee experiment (N^0 11) could not be confirmed during later testings. This result may be explained by external contamination of viroids on the leaves and/or (cross-)contamination during the analysis in the lab (plant preparation, RNA extraction, cDNA synthesis or PCR).

While the infection through infected pollen that germinates on the receiving plant stigma could explain the observed intraspecific transmission of pospiviroids or viruses in tomato another mechanism is likely to be involved in interspecific transmission (Antignus *et al.* 2007, Matsuura *et al.* 2010, Shipp *et al.* 2008). For instance, the necessary entry point for viroid inoculation could occur when bumblebee mandibles become contaminated with infected plant sap during nectar robbing or flower biting observed in the so-called 'buzz pollination' of tomato (Antignus *et al.* 2007, Matsuura *et al.* 2010, Shipp *et al.* 2008).

It is worth noting that the only transmission event of this study occurred in the experiment performed at the highest temperature applied (25°C). Because pospiviroid concentration and mechanical transmissibility seem to increase with temperature (Harris & Browning 1980, Schuman *et al.* 1980), we postulate that the risk of transmission by insects increases accordingly. Interestingly, also in the literature, successful pospiviroid insect transmission without heterologous encapsidation has been reported at day temperatures equal to or greater than 25°C (Antignus *et al.* 2007, De Bokx & Piron 1981, Matsuura *et al.* 2010, Schuman *et al.* 1980).

In conclusion, out of twelve experiments with three commonly used or encountered insect species in susceptible crops, only one TCDVd transmission event was recorded, i.e. when bumblebees were applied. Considering the high density of bumblebees used, the close proximity of the infection source and the relatively low transmission efficiency in tomatoes (1/39 = 2.6%), it is concluded that pospiviroid transmission by bumblebees is possible, but the risk is low. This conclusion is supported by the observation of widespread pospiviroid outbreaks in ornamental plants in Europe, whereas relatively few outbreaks are reported in susceptible crops such as tomato and pepper. It is thus our opinion that pollinating insects and biological control agents used in these susceptible crops are not a major phytosanitary threat for viroid dispersal.

Chapter 5:

Assessment of bumblebee health after pospiviroid ingestion

5.1 INTRODUCTION

Since the beginning of the nineties, several companies started commercially rearing bumblebees (*B. terrestris*) for large-scale greenhouse pollination purposes, such as the pollination of greenhouse tomatoes (Goulson 2010). Similar to bees occurring in the wild, these domesticated bumblebees are hosts to multiple RNA viruses (McMahon *et al.* 2015, Piot *et al.* 2015). Apart from bee-specific viruses, bumblebees also get into contact with plant viruses by interacting with infected plants and foraging on their nectar and pollen. This close contact between plant viruses and bees can in some cases lead to pollen-mediated virus transmission, where pollen is carried in the pollen basket ("corbicula", Figure 5.1) on the hind legs of the bee and disseminated while visiting plants.

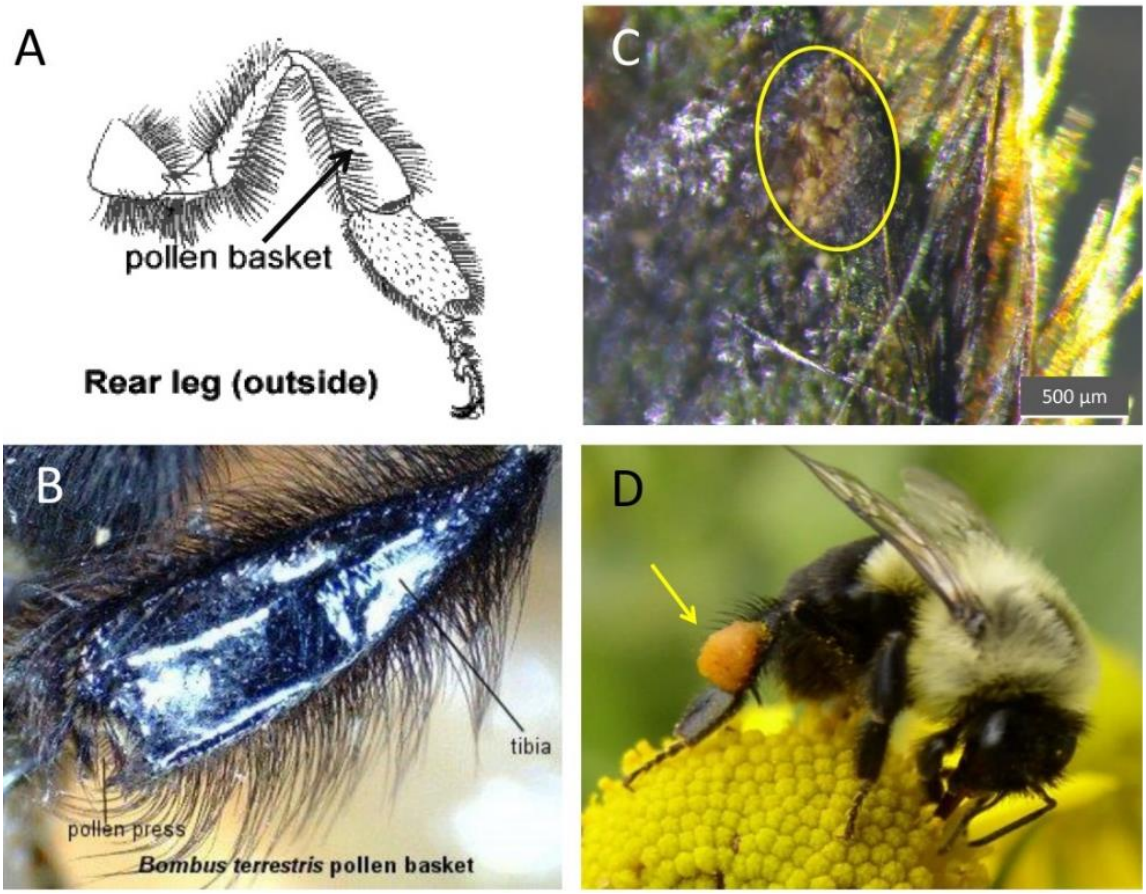


Figure 5.1: A) Black arrow = pollen basket on the rear leg of a bumblebee (Image obtained from: http://www.bumblebee.org/ 22-1-2016). The pollen basket is a flat, shiny area on the leg which is surrounded by brittle hairs on the sides, B) Microscopic image showing the pollen press and tibia (Image obtained from: http://www.bumblebee.org/ 22-1-2016), C) Yellow circle = small concentration of pollen on the corbicula , D) Yellow arrow = pollen cluster on the hind leg of a female bumblebee (Image obtained from: https://polinizador.wordpress.com/ 22-1-2016).

Examples of pollen-mediated plant virus transmission are *Pepino mosaic virus* (PMV) (Shipp *et al.* 2008), *Blueberry shock ilarvirus* (BlShV) (Bristow & Martin 1999) and potentially certain pospiviroids, like TCDVd and TASVd (Chapter 4, Antignus *et al.* 2007, Matsuura *et al.* 2010). For pollen-vectored plant viruses, the virions can be located both inside and outside the pollen grains (Singh *et al.* 2010).

In Chapter 4, it was demonstrated that bumblebees actively visit pospiviroid-infected flowers in transmission experiments and are able to transmit pospiviroids to receiver plants. Recently, it was observed that the plant virus *Tobacco ringspot virus* (TRSV) was able to infect bees after exposure to TRSV-contaminated pollen (Li *et al.* 2014). This illustrates that apart from insect viruses, also plant viruses may contribute to pollinator declines. In the study of Li *et al.* (2014) effects of TRSV on honeybees were assessed by monitoring 10 colonies for a period of 1 year, starting in March and finishing in February of the following year. In this Chapter, we will assess whether TASVd is detected in bumblebee progeny after feeding on TASVd-contaminated pollen and if bumbleblees are experiencing any health effects as a result of this feeding. To investigate this two feeding experiments were organized, in which bumblebees in microcolonies were fed throughout a period of 50 days with *Tomato apical stunt viroid* (TASVd)-contaminateds pollen. During and at the end of the experimental period all lifecycle parameters were counted and differences between treatments were explored statistically.

5.2 MATERIAL AND METHODS

5.2.1 Inoculation and testing of *Petunia*

As an inoculum source, ten petunias were used, after they had been successfully inoculated with a TASVd-isolate from *S. jasminoides* (GenBank Accession N^0: KF484878) as described in Chapter 2 (Verhoeven and Roenhorst 2000). After 4 weeks, RNA was extracted from petunia leaves (1 leaf for each of the 10 plants) and pollen (±10 mg for each plant) using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA). Specific TASVd detection was done using the Agpath-ID™ one-step RT qPCR Kit (Applied Biosystems, Foster City, CA, USA) and the primers and probe of Monger *et al.* (2010).

5.2.2 Stability of TASVd-detection in different matrices

In preparation of the bumblebee feeding (see 5.2.3) , it was important to first assess the (stability of) detection of TASVd in a pollen and sugarwater matrix. This was determined in a time-lapse experiment of 1 week. A 1:5 mixture of TASVd RNA and sugarwater (Biogluc®, Biobest, Westerlo, Belgium) was divided over eight Eppendorf tubes, containing a final volume of 100 µl each. In addition, eight Eppendorf tubes were filled with 100 µl sugarwater to which 1 g of TASVd-infected pollen was added. As a control, eight tubes with a 1:5 mixture of TASVd and tap water were used. Next, these 24 tubes were maintained in the lab at room temperature (21°C). After various time periods (30 minutes, 1h, 2h30, 12h, 2, 3, 4 and 7 days), RNA was extracted from one Eppendorf tube of each matrix (sugarwater, pollen and tap water) with the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA). RT-qPCR analysis was done with a one-step RT-qPCR (Agpath-IDTM one-step RT qPCR Kit, Applied Biosystems, Foster City, CA, USA) and the primers and probe of Monger *et al.* (2010).

5.2.3 Experimental design of the feeding experiments

For the first feeding experiment (Experiment 1) 40 worker bees (Biobest, Westerlo, Belgium) were randomly divided over 8 plastic nestboxes (Ø 9cm). At the bottom of each nestbox, a plastic container with sugarwater (Biogluc®, Biobest, Westerlo, Belgium) was attached. A cottonwool straw in this container permitted bumblebees to drink *ad libitum*. All 8 nestboxes were arranged onto a table which was placed in a climate room (average temperature = 27°C, humidity = 45%, according to Figure 5.2).

During 50 days, microcolonies were followed and all life stages (workerbees, eggs, larvae, pupae, drones) and sugarpots were counted. The experiment was repeated with a new batch of bumblebees one month later in exactly the same way (Experiment 2).

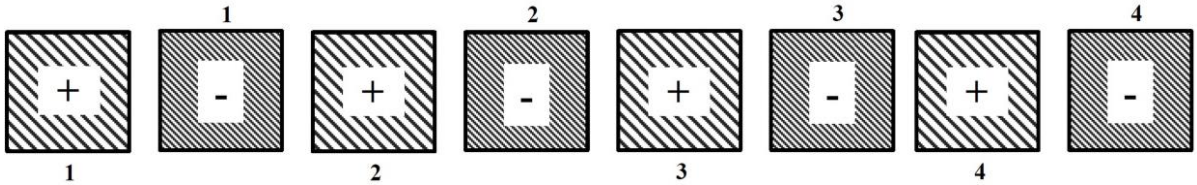


Figure 5.2: Experimental set-up of the feeding experiments with 4 contaminated (+) and 4 non-contaminated (-) microcolonies.

5.2.4 Preparation of the pollen for the feeding experiments

Each microcolony was given 2 g of contaminated or non-contaminated pollen (Biobest, Westerlo, BE) at the beginning of the week (i.e. Monday) and at the end of the week (i.e. Friday) during the course of the experiments (50 days). For the non-contaminated colonies, the pollen mixture was kneaded with 500 µl of sugarwater. The same was done for the contaminated colonies, only here the 500 µl sugarwater contained pollen that was assembled from TASVd-infected *Petunia flowers* (± 2 mg). The pollen of these flowers was dusted on a Petridish that was placed under a stereomicroscope and consequently mixed with 500 µl sugarwater.

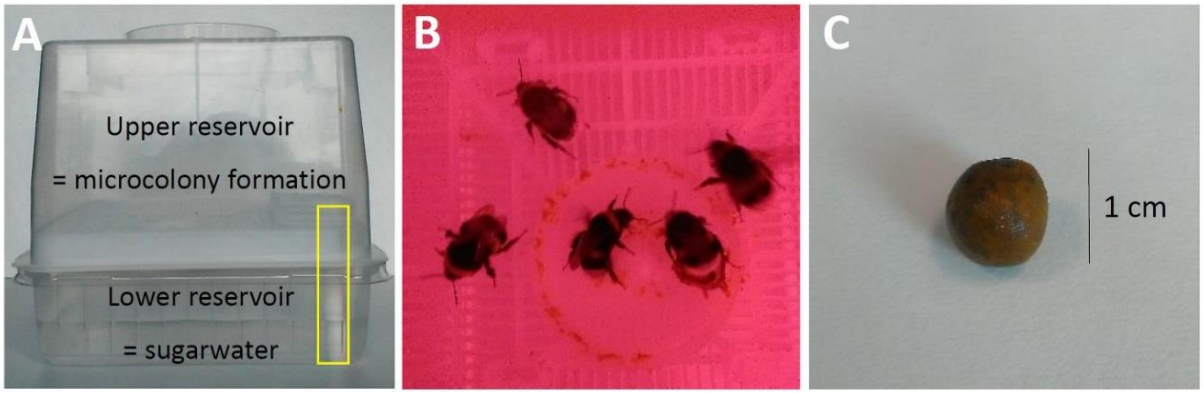


Figure 5.3: A: Example of a nestbox with a lower reservoir containing sugarwater (yellow rectangular = straw that connects the lower and upper reservoir) and an upper reservoir for microcolony formation, B: A picture of 5 worker bees just after settlement, C: a ball of kneaded pollen that was provided to each one of the microcolonies twice a week.

5.2.5 RNA-extraction of bumblebee lifestages

After 50 days, all nestboxes were placed in freezer (-20°C) for 4 hours. After counting the numbers of specimens in each of the life stages and the number of sugarpots in each colony, the dronal weights were measured. From each colony, one sample of each life stage was taken (i.e. 1 worker bee, 1 egg, 1 larva, 1 pupa, 1 drone) and RNA was extracted from these samples using the RNeasy Mini Kit (Qiagen, Hilden, Germany). A volume of 1.5 ml RLT buffer RNeasy Mini kit (Qiagen, Hilden, Germany) was used to start the RNA-extraction. After centrifuging this mixture at 14.000 rpm, 500 µl of the supernatans was used to continue the extraction procedure according to the manufacturer's conditions. For workerbees and drones, the guts and mouthparts were dissected. After RNA-extraction, cDNA was made using the iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). qPCR detection was done using the SensiFast™ Probe Hi-Rox kit (Bioline Reagents, London, UK) and the generic pospiviroid-probe and primers of Botermans *et al.* (2013).

5.2.6 Statistical analysis

Our dataset was analyzed in R-Studio (version 0.99.902) and STATISTICA (version 12) software (Statsoft, Tulsa, Oklahoma). Linear Mixed-Effects (LME) models with "Treatment" as a fixed factor and "Experiment" as a random factor were constructed and Student's T-tests were used for two group comparisons.

5.3 RESULTS

5.2.1 Test results of the infected *Petunia* plants

After 4 weeks, leaves and pollen of ten TASVd-infected petunias resulted in a mean Cq-value of 21 ± 0.5 for the 10 leaf samples and a mean Cq-value of 22 ± 0.12 for the 10 pollen samples.

5.3.1 Stability of TASVd-detection in different matrices

Table 5.1 shows the RT-qPCR detection results of samples containing mixtures of TASVd and tap water, sugarwater or pollen that were RNA-extracted and analyzed after eight different points in time. The mean of these eight time-lapse samples is 20.8 ± 0.93 for tap water, 22.7 ± 1.18 for sugarwater and 22.4 ± 0.67 for the pollen samples (Table 5.1). Hence, TASVd remained stable over the period of 7 days in the three matrices.

Table 5.1: Mean Cq-values for each sample (Sample N^{o}) of tap water, sugarwater and pollen and its technical replicate ± Standard Deviation (SD). "Time" represents the number of minutes/hours/days after the start of the experiment at which each sample was processed for RNA-extraction and RT-qPCR detection.

Sample N^{o}	Time	Cq (tap water)	Cq (sugarwater)	Cq (pollen)
1	30 min	20.30 ± 0.28	22.50 ± 0.57	21.94 ± 0.59
2	1h	18.97 ± 0.18	23.94 ± 0.20	21.08 ± 0.11
3	2h30	21.03 ± 0.11	24.81 ± 0.27	22.39 ± 0.64
4	12h	20.75 ± 0.07	22.22 ± 1.58	22.68 ± 0.32
5	2 days	20.26 ± 0.83	21.37 ± 0.59	22.92 ± 0.10
6	3 days	21.41 ± 0.13	22.90 ± 0.28	22.38 ± 0.03
7	4 days	21.32 ± 0.47	21.87 ± 0.34	23.1 ± 0.42
8	7 days	22.00 ± 0.71	21.73 ± 0.38	22.98 ± 0.18

5.3.2 First appearances of each lifecycle parameter

The number of days until the first appearance of eggs, pupae and drones was similar for the non-contaminated and the contaminated treatments of both experiments I and II (Table 5.2). The mean number of days for each of the life stages was not significantly different between the contaminated and the non-contaminated treatments (LME, Student's t-test, p-values >0.05, α =0.05).

Table 5.2: Mean number (N^0) of days ± standard deviation (SD) until first appearance of the eggs, pupae and drones for both experiments 1 and 2. C = contaminated, NC = non-contaminated.

Experiment	Treatment	Life stage	Mean N^0 of days ± SD
1	C	Eggs	8 ± 0.50
		Pupae	22 ± 0.95
		Drones	34 ± 1.50
	NC	Eggs	8 ± 0.50
		Pupae	21 ± 0.50
		Drones	34 ± 1.73
2	C	Eggs	9 ± 0.82
		Pupae	23 ± 0.58
		Drones	35 ± 0.00
	NC	Eggs	9 ± 0.50
		Pupae	23 ± 0.58
		Drones	35 ± 0.00

5.3.3 Lifecycle parameters on day 51

Table 5.3 provides an overview of the values for each of the lifecycle parameters (N^0 of workerbees, eggs, larvae, pupae and drones) at the end of the experiment.

Table 5.3: The number of workerbees, eggs, larvae, pupae, drones, mean dronal weight (MDW, in g) and Total Biomass (MDW*N^0 of drones, in g) on day 51 for non-contaminated (NC) and contaminated (C) microcolonies after the start of the first (1) and second (2) fitness experiment.

Experiment	Treatment	Microcolony	N^0 workerbees	N^0 eggs	N^0 larvae	N^0 pupae	N^0 drones	MDW	Total Biomass (g)
1	C	1	5	92	15	11	24	0.32	7.77
		2	5	7	30	/	34	0.32	10.94
		3	4	26	39	13	27	0.35	9.52
		4	4	23	11	15	26	0.34	8.84
	NC	1	5	27	18	12	25	0.37	9.26
		2	5	45	83	16	18	0.32	5.78
		3	4	70	47	11	30	0.36	10.86
		4	5	73	34	5	25	0.36	8.98
2	C	1	5	28	73	16	27	0.34	9.15
		2	5	25	42	15	27	0.35	9.36
		3	5	10	50	6	34	0.28	9.52
		4	5	18	35	6	30	0.37	10.97
	NC	1	5	40	41	16	34	0.35	11.85
		2	5	33	27	9	25	0.31	7.69
		3	5	14	8	20	16	0.35	5.55
		4	5	56	42	16	24	0.35	8.48

The number of drones that was generated by the end of the experiments, was similar for the non-contaminated and contaminated treatments (Table 5.3). In contrast, the number of eggs fluctuated a lot within and between treatments (Table 5.3, Figure 5.4). However, statistical testing did not result in any significant differences in any of the lifestages between the infected and non-infected treatments of experiment I and II on day 51(LME, Student's t-test, p-value Eggs = 0.18, p-value Larvae = 0.95, p-value Pupae = 0.52, p-value Drones = 0.12, α =0.05).

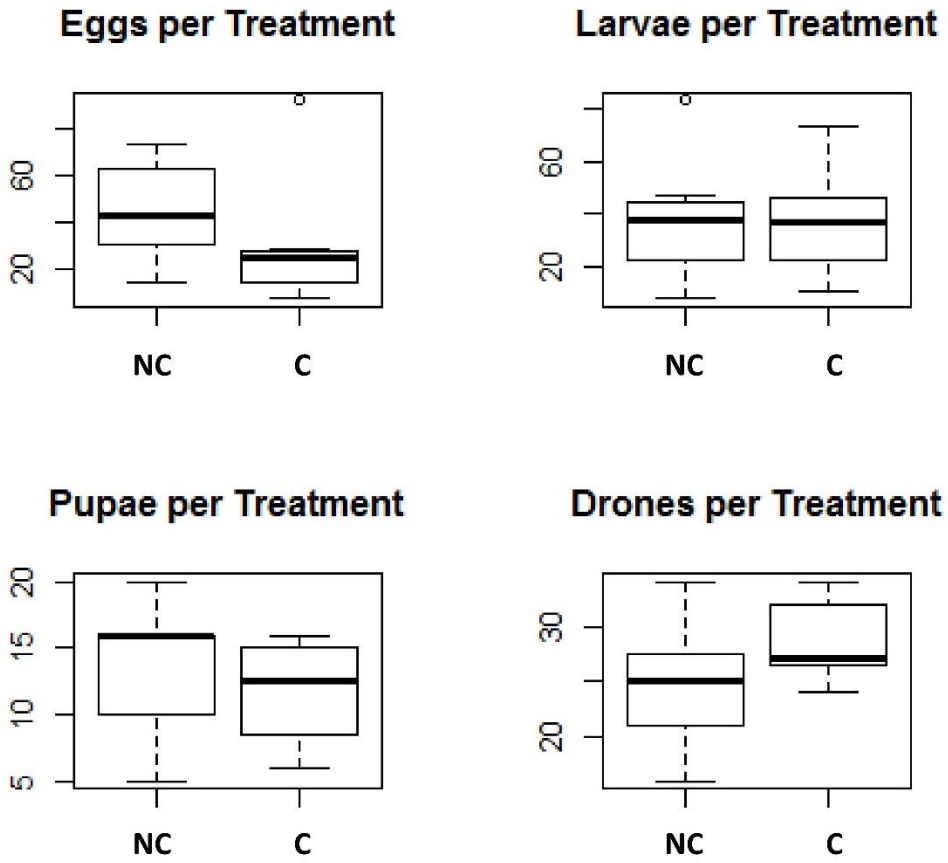


Figure 5.4: Boxplots of pooled eggs, larvae, pupae and drones for the contaminated (C) and non-contaminated (NC) microcolonies of both experiment I and II 51 days after the start .

5.3.4 Total Biomass

The Total Biomass per microcolony at day 51 was calculated as the number of drones on day 51 ("N° Drones", Table 5.3) times the mean dronal weight (MDW, Table 5.3) on day 51. There is no significant difference (LME, Student's t-test, p-value 0.256 > 0.05, α =0.05) in Total Biomass between the contaminated and non-contaminated treatments.

5.3.5 Evolution of the lifecycle parameters over the experimental period

The response of the workers was regularly checked by gently shaking the microcolony so that "healthy" workers would instantly fly up (Mommaerts *et al.* 2010) and no abnormalities in behavior were recorded. The evolution of the lifecycle parameters over the experimental period was assessed by calculating "weekly means" and plotting these on a time axis.

Figure 5.5 shows the evolution of the mean number of sugarpots and pupae on a weekly basis for Experiment 1. The weekly mean sugarpots and pupae did not significantly differ between the two treatments over the experimental period (LME, Student's t-test, p-value = 0.344 > 0.05, α =0.05).

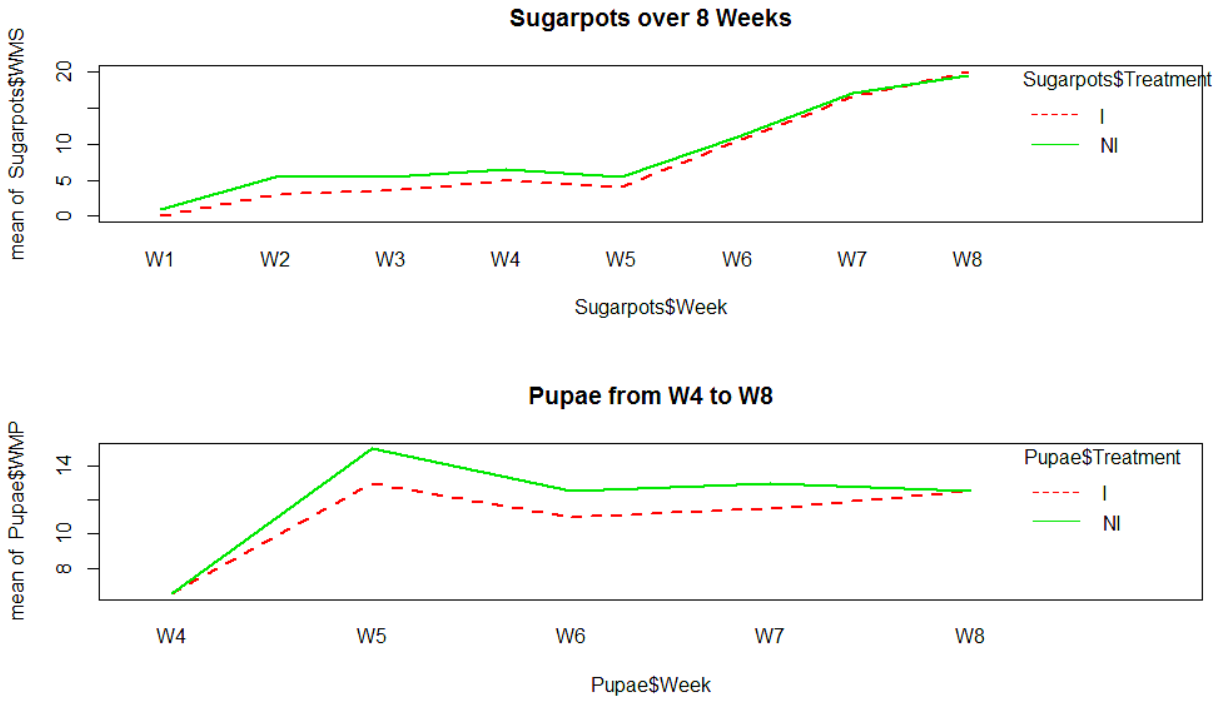


Figure 5.5: The evolution of the N^0 (number) of Sugarpots (upper panel) and Pupae (lower panel) over time for Experiment I. Red (I) = contaminated, Green (NI) = non-contaminated.

5.3.6 Testing of the lifecycle parameters for pospviroids

None of the lifecycle stages sampled on day 51 that were tested for pospiviroids using a two-step qPCR gave positive detection results for TASVd.

5.4 DISCUSSION

RT-qPCR detection of ten TASVd-infected *Petunia* plants resulted in similar detection levels for leaves and pollen. This has also been observed for PSTVd, where no differences were noted in concentration between PSTVd-infected *S. jasminoides* leaves and pollen after RT-qPCR detection (TOPOVIR 2011). Detection levels of TASVd remained quite stable in three different matrices (pollen, sugarwater and tap water) over the different test points (from 30 minutes up to 7 days after the start of the experiment). The observed stability of TASVd in various matrices is in line with previous results acquired for PSTVd (Mackie *et al*. 2015, Mehle *et al.* 2014, Verhoeven *et al.* 2010c).

After stability of TASVd in pollen had been confirmed, bumblebee microcolonies were fed with TASVd-contaminated and non-contaminated pollen for a period of 50 days. None of the lifestages that were tested 50 days after the start of the experiment, showed signs of pospiviroid presence, indicating that viroids were not surviving in the insect bodies, or their progeny, throughout the experimental period. Hence, there is no reason to assume that viroids are able to replicate in bumblebees. Additionally, no relevant differences were noted between the microcolonies feeding on contaminated and non-contaminated pollen during the daily visual inspection of the microcolonies. Also the development of each of the lifestages appeared to be similar between the contaminated and control treatments. This was confirmed by statistical analyses showing a similar appearance and development of eggs, larvae, pupae and a similar total biomass after 50 days. For future experiments, it could be interesting to count the number of dead larvae per day and calculate the larval weight at the end of the feeding period, since these parameters were not assessed.

While statistically no significant differences could be detected between the non-contaminated and the contaminated microcolonies of the feeding experiments, minor differences between the two treatments were observed. These slight differences in lifecycle parameters between the two treatments may be explained by biological variations between microcolonies. It is concluded for this Chapter, that TASVd is not detected in bumblebee progeny after prolonged feeding on TASVd-contaminated pollen. Additionally, bumblebees feeding on this pollen do not suffer from any negative effects since infected (micro)colonies developed similarly as in the healthy controls.

Chapter 6:

A new look at pospiviroid-luteovirus associations

6.1 INTRODUCTION

Co-infections of plant-pathogenic viruses and viroids have been repeatedly reported worldwide (Herranz *et al.* 2013, Karosawa & Ehara 1988, Kondakova *et al.* 1989, Querci *et al.* 1997, Syller *et al.* 1997). Some of these studies presented evidence that viroids can be transmitted together with viruses, and their respective vectors (such as aphids) to receiver plants, if source plants are doubly inoculated with both (Francki *et al.* 1986, Querci *et al.* 1997; Salazar *et al.* 1995, Syller *et al.* 1997). This observation was explained by the phenomenon of "transencapsidation", defined as the encapsidation of the nucleic acids of a virus or viroid into the virion of another virus. Transencapsidation of viroids in viruses, and subsequent transmission through insects, could potentially have important epidemiologic implications: a latently present viroid of a given crop could be incorporated into the virion of a plant virus (e.g. into the icosahedral capsid of a luteovirus) and subsequently be transmitted by an insect vector (e.g. an aphid, Francki *et al.* 1986). This pathway of transencapsidation, followed by vector-mediated transport, could result in the infection of another host plant and evoke the viroid symptoms that were not expressed in the former host (Francki *et al.* 1986).

Until this date, transencapsidation was investigated for one only viroid species (*Potato spindle tuber viroid*, PSTVd) and three candidate viruses: *Potato leafroll virus* (PLRV, Querci *et al.* 1997, Salazar *et al.* 1995, Syller *et al*. 1997, Syller & Marczewski 2001), *Velvet tobacco mottle virus* (VTMoV) (Francki *et al.* 1986) and *Potato virus Y* (PVY) (Singh *et al.* 1992a). Most of these studies focused on *Luteoviridae* (like PLRV) as potential viroid carriers (Querci *et al.* 1997, Salazar *et al.* 1995, Syller *et al.* 1997, Syller & Marczewski 2001) since for these viruses transencapsidation of small RNAs has been observed frequently before (Falk *et al.* 1995). Indeed, the size (25-30nm) and icosahedral shape of luteovirid virions has potential to accommodate alien small RNAs (Falk *et al.* 1995). Francki *et al.* (1986) showed that PSTVd-RNA can get transencapsidated by VTMoV in vitro. However, in the study of Singh *et al.* (1992a) it was concluded that transencapsidation did not take place for *Potato virus Y* (PVY). This study suggested the possibility of PSTVd-infection due to outside contamination of the PVY particles by the viroid (Singh *et al.* 1992a). Later, in experiments by Salazar *et al.* (1995) 100% transmission of PSTVd was achieved from doubly inoculated PLRV and PSTVd plants and no transmission was observed when source plants were infected with only the viroid.

Additionally, Querci *et al.* (1997) were able to still detect viroids after virus purification and Micrococcal nuclease (MNase) blasting and therefore concluded that viroids must have been protected from enzymatic degradation because they were encapsidated. MNase is an exo/endonuclease derived from *Staphylococcus aureus* that degrades both DNA and RNA in linear/circular and single-stranded/double-stranded form resulting in the formation of 3' phosphomono- and dinucleotides (Horz & Altenburger 1981). It cleaves preferentially in AT or AU-rich sequences and has an optimal activity around a pH of 9.2 for both RNA and DNA substrates (Horz & Altenbruger 1981).

The studies of Francki *et al.* (1986), Querci *et al.* (1997), Salazar *et al*. (1995) and Syller *et al.* (1997) share the same biochemical methodology: viral purification from doubly infected plants via ultracentrifugation and consequently, the use of nucleases to degrade non-encapsidated viroids in purified samples. Testing of the remaining virus and viroids to confirm encapsidation was accomplished via serological (ELISA) and molecular assays (dot blot/PAGE/RT-PCR). However, there are still some unresolved issues concerning these findings: 1) until now, there is no direct or visual proof of transencapsidation, 2) nuclease efficiency has been insufficiently assessed, 3) insensitive molecular detection techniques were employed, 4) no ultrastructural knowledge on where the viroid could be located within the virus particle, 5) only one viroid species (PSTVd) has been investigated. Therefore, more research is needed to be sure that the viroids were indeed encapsidated and not, for instance, attached to the outside of the viral particle.

In this Chapter, two tests that were crucial in delivering evidence for transencapsidation are evaluated for two pospiviroid species that are closely related to PSTVd, namely TCDVd and TASVd. First, MNase activity was assessed by testing two enzyme concentrations on purified TCDVd and PLRV mixtures. Remaining viral and viroid RNA was detected using RT-qPCR. Second, transmission experiments with the green peach aphid (*M. persicae*) as a PLRV-vector were organized for TCDVd and TASVd. The results of these and previous experiments are discussed and directions for future research on this topic are provided.

6.2 MATERIAL AND METHODS

6.2.1 Inoculation, propagation and testing of the host plants

A stock of potato plants (*S. tuberosum* cv. Kennebec) infected by PLRV (GenBank Accession N^0 = KX364206) was maintained in a climate chamber (3x2 m, average temperature 23°C). New PLRV-infected potatoes were obtained by aphid-assisted transmission (*M. persicae*). Each of the plants was regularly tested for PLRV-infection by testing newly emerging leaves with RT-qPCR. From the leaves RNA was extracted using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA). Detection was done using the Agpath-ID^{TM} one-step RT qPCR Kit (Applied Biosystems, Foster City, CA, USA) and specific PLRV-primers and probe (Agindotan *et al.* 2007, Table 6.1).

Seed potato tubers (cv. Kennebec, certification class "E2") were supplied by the Diagnostic Center for Plants (DCP, ILVO) after these had tested negative for Potato Virus X, Y and PLRV by PCR. The potato plants that developed from these tubers were checked for symptoms and tested with the generic primers of Olivier *et al.* (2014) for pospiviroid infections. Plants that did not develop any viroid/virus-related symptoms and that tested negative for pospiviroids, were selected for inoculation. Ten plants were mechanically inoculated with TASVd (GenBank Accession N^0: KF484879) and another batch of ten with TCDVd (GenBank Accession N^0: KU714934) using the inoculation procedure as described in Verhoeven and Roenhorst (2000). Host plants were tested for these viroids using specific (Boonham *et al.* 2004) and generic (Botermans *et al.* 2013) RT-qPCR primers and probes (Table 6.1). For RT-PCR amplification with the Olivier *et al.* (2014) primers, cDNA was synthesized using the iScriptTM cDNA synthesis kit (Bio-Rad, Hercules, CA, USA).

Table 6.1: (q)RT-PCR primers and probes for the detection of PLRV, TASVd and TCDVd.

Target	Name	Primer type	Sequence (5'-3')	Reference
PLRV	PLRV103-5FP	FW	AAAGCCGAAAGGTGATTAGGC	Agindotan *et al.* 2007
	PLRV103-5RP	RE	CCTGGCTACACAGTCGCGT	
	PLRV103-5	Probe	CTCAACGCCTGCTAGAGACCGTCGAAA	
TASVd TCDVd	TCR-F 1-1	FW	TTCCTGTGGTTCACACCTGACC	Botermans *et al.* 2013
	TCR-F 1-3	FW	CCTGTGGTGCTCACCTGACC	
	TCR-F 1-4	FW	CCTGTGGTGCACTCCTGACC	
	TCR-F PCFVd	FW	TGGTGCCTCCCCCGAA	
	TCR-F IrVd	FW	AAT GGTTGCACCCCTGACC	
	TR-R1	RE	GGAAGGGTGAAAACCCTGTTT	
	TR-R CEVd	RE	AGGAAGGAGACGAGCTCCTGTT	
	TR-R6	RE	GAAAGGAAGGATGAAAAT CCTGTTTC	
	pUCCR	Probe (6FAM/MGB)	CCGGGGAAACCTGGA	
TCDVd	PSTV-231F	FW	GCCCCCTTTGCGCTGT	Boonham *et al.* 2004
	PSTV-296R	RE	AAG CGGTTCTCGGGAGCTT	
	PSTV-251T	Probe	CAGTTGTTTCCACCGGGTAGTAGCCGA	
TASVd TCDVd	Pospi1deg-FW	FW	GGGAKCCCCGGGGMAAC	Olivier *et al.* 2014
	Pospi1s-RE	RE	TCAGTTGTWTCCACCGGGT	

6.2.2 MNase tests

Activity of Micrococcal Nuclease (MNase) from *Staphylococcus aureus* (Mw= 16,807 g/mol, Sigma-Aldrich, St. Louis, MO, USA) was tested for two enzyme concentrations. In two biological replication tests, MNase was applied to six types of samples (Table 6.2).

Table 6.2: Treatment and content description of the six types of samples used in the MNase-tests.

Treatment	Description
1. Healthy	RNA extracted from a healthy potato
2. PLRV Virus	Purified PLRV-virus from a PLRV-infected potato
3. PLRV RNA	RNA extracted from a PLRV-infected potato
4. PLRV + TCDVd	Purified PLRV-virus from a doubly-infected potato
5. TCDVd	RNA extracted from a TCDVd-infected potato
6. 1:1000 TCDVd	1/1000 dilution from Treatment "TCDVd"

In these two tests, the same original plant material was used (see 6.2.1) but RNA-extractions and PLRV purifications were executed independently from each other.Purified PLRV-particles (Table 6.2) were obtained through enzyme-assisted (Driselase) purification and ultracentrifugation, following the protocol of Takanami and Kubo (1979). Final purified pellets were tested for TCDVd and PLRV using RT-qPCRs (as described above) and PLRV capsid protein detection was determined by Doubly antibody sandwich (DAS) enzyme-linked immunosorbent assay (ELISA) (Bioreba, Reinach, Switserland).

Samples of Table 6.2 were treated with two concentrations of MNase: 0.001 U/µl concentration of MNase (i.e. the conditions of Querci *et al.* 1997) and 0.01 U/µl. Apart from the MNase solution, 0.1 M $CaCl_2$ solution was added to each vial to start the reaction. Vials were then transferred to a thermomixer with a temperature of 28°C and incubated for 15 minutes. To stop the reaction, vials were placed on ice and 0.1 M EDTA was added. After two minutes of incubation, RNA was extracted from each of the vials using the RNeasy Mini Kit (Qiagen, Hilden, Germany) whilst maintaining a temperature of 4°C in the centrifuge throughout the extraction procedure. Detection of the viroid and viral RNA was then done using the RT-qPCR detection kits (as described before) with primers from Agindotan *et al.* (2007) for PLRV-detection and Boonham *et al.* (2004) for TCDVd detection (Table 6.1).

6.2.3 Transmission experiments

In the first transmission test with TCDVd, twenty adult apterous *M. persicae* were placed onto four different treatments of source plants for an Acquisition Period (AP) of two days. The four treatments were: “non-infected”, “PLRV + TCDVd”, “PLRV” and “TCDVd”. The source plants were seedlings of *N. benthamiana* and *S. tuberosum* (cv. Kennebec). For each treatment two plants of each species were placed in separate gauze cages (60 x 60 x 90 cm, mesh size=0.8 × 0.8 mm). The cages were placed inside a climate chamber (3 x 2 m, average temperature 23°C). After the AP, two *N. benthamiana* and *S. tuberosum* receiver plants were placed inside the cage, whilst avoiding physical contact between source and receiver plants. After two weeks, and every week hereafter, newly formed leaves from each of the source plants were sampled and tested for PLRV using the RT-qPCR primers of Agindotan *et al.* (2007) and for TCDVd using the RT-qPCR primers of Botermans *et al.* 2013 (Table 6.1).

In a second experiment with TASVd, two rows of five doubly inoculated (TASVd and PLRV) potato plants (cv. Kennebec) were planted in the soil of a greenhouse plot (5x10m). Twenty adult apterous *M. persicae* were placed onto each source plant. After two weeks non-infected potato plants ("receiver plants") were planted in between the rows of infected plants keeping 1 m distance between each row. Potato stems were attached to supporting sticks in order to prevent physical contact between source and receiver plants. After 2 weeks, and every week hereafter, receiver plants (leaves and potato tubers) were tested for PLRV with primers of Agindotan *et al.* (2007) and for TASVd with the primers of Botermans *et al.* (2013).

6.3 RESULTS

6.3.1 MNase tests

For each of the six MNase-test treatments as described in Table 6.2, RT-qPCRs were conducted for PLRV (Agindotan *et al.* 2007) and TCDVd (Boonham *et al.* 2004) (Table 6.3). The PLRV RNA and viroid concentration decreased after MNase digestion, but considerable variation was observed within treatments and between the two repetitions of the experiment. However, in several cases the concentration of 0.001 U/µl MNase was not sufficient to degrade all viroid RNAs present in the sample (Cq-values ≤ 35). When using a concentration ten times more concentrated (0.01 U/µl), viroid RNA was detected in various cases (at a cut-off Cq-value of 35).

Table 6.3: Overview of the Cq-results after PLRV and TCDVd RT-qPCRs of the six sample types in the MNase tests. Mean Cq-values ± Standard Deviation before MNase (Before MN) and after MNase (After MN) treatment were calculated after two repetitions of the experiment. ND = no detection result.

Concentration (U/µl)	Treatment	PLRV		TCDVd	
		Before MN	After MN	Before MN	After MN
0,001	Healthy	ND	ND	ND	ND
	PLRV RNA	17 ± 0.0	23 ± 7.1	ND	ND
	PLRV virus	30 ± 4.3	32 ± 5.0	ND	ND
	PLRV + TCDVd	30 ± 5.7	32 ± 7.1	27.5 ± 5.0	32 ± 6.4
	TCDVd	ND	ND	24 ± 4.2	33 ± 5.7
	TCDVd 1:1000	ND	ND	28 ± 8.5	39 ± 0.4
0,01	Healthy	ND	ND	ND	ND
	PLRV RNA	17 ± 0.7	31 ± 10	ND	ND
	PLRV virus	34 ± 0.0	38 ± 2.1	ND	ND
	PLRV + TCDVd	35 ± 2.8	37 ± 0.0	28 ± 6.4	36 ± 1.4
	TCDVd	ND	ND	25 ± 5.0	34 ± 6.0
	TCDVd 1:1000	ND	ND	30 ± 5.3	ND

In addition to the RT-qPCR according to Boonham *et al.* (2004) also an RT-PCR with the primers of Olivier *et al.* (2014) was performed on these samples (Figure 6.1).

Figure 6.1 shows the results of the six identical treatments after cDNA synthesis, RT-PCR and capillary electrophoresis. Clear bands at the expected positions were obtained in lanes 4-6 before MNase applications were performed for both concentrations (Figure 6.1 A - B). After application of MNase, two bands were observed (lanes 10 and 11) when 0.001 U/µl MNase was applied, whereas no clear amplicons were obtained for 0.01 U/µl (Figure 6.1 B). This observation was confirmed after checking the electropherograms of each lane.

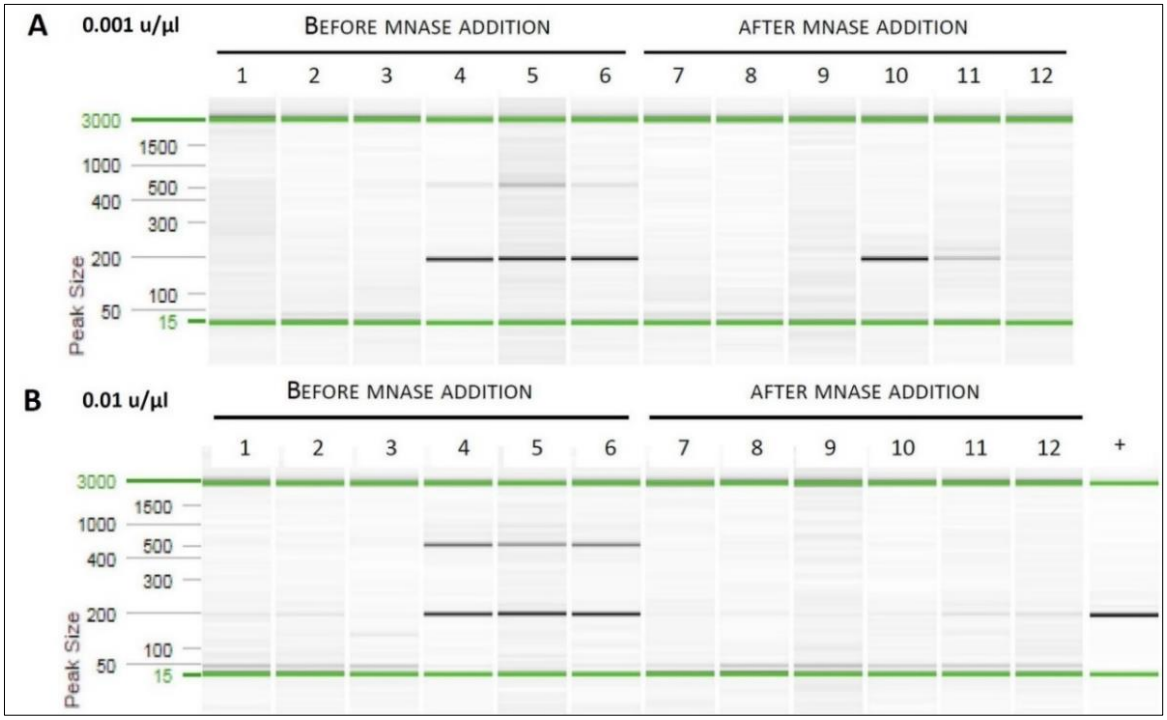


Figure 6.1: Capillary electrophoresesis results after TCDVd detection (Olivier *et al.,* 2014) showing the six treatments (Table 6.3) before and after MNase digestion for the first MNAse experiment that was performed. A) Concentration MNase = 0.001 U/µl, B) Concentration MNase = 0.01 U/µl, "+" = positive TCDVd cDNA control. 1-6: six treatments (Healthy, PLRV Virus, PLRV RNA, PLRV + TCDVd, TCDVd and 1:1000 TCDVd) before MNase addition, 7-12: six treatments (Healthy, PLRV Virus, PLRV RNA, PLRV + TCDVd, TCDVd and 1:1000 TCDVd) after MNase addition.

6.3.2 Transmission experiments

In the two types of transmission experiments (gauze cages vs. greenhouse) neither TCDVd nor TASVd was transmitted in conjunction with PLRV. In the experiment with the gauze cages only PLRV was successfully transmitted to the receiver plants (Table 6.4). This shows that aphid-assisted transmission was only successful for the virus, and not for the viroid. During this experiment the "non-infected" control treatment remained non-infected.

Table 6.4: Number of infected receiving plants on the total number of receiving plants (*N. benthamiana* and *S. tuberosum*) 6 weeks after placing them in the gauze cage with the infected source plants and aphids. Healthy = non-infected host plants.

Host	Treatment				
	PLRV	PLRV + TCDVd		TCDVd	Healthy
N. benthamiana	2/2	2/2	0/2	0/2	0/2
S. tuberosum	2/2	2/2	0/2	0/2	0/2

Also in the greenhouse experiments, the twenty receiving potato plants that were planted 1 m away from doubly infected (PLRV + TASVd) source plants did not get infected with TASVd. Only PLRV was successfully transmitted to the receiver plants.

6.4 DISCUSSION

In this study, the efficiency of the enzyme MNase in degrading TCDVd RNA was evaluated for two concentrations: 0.001 U/µl and 0.01 U/µl. Our results show that the MNase conditions employed by Querci *et al.* (1997) are insufficient to degrade TCDVd completely. In the experiments of Querci *et al.* (1997) the concentration 0.001 U/µl was used, in combination with an incubation of 15 minutes at 30°C, stating also that trial experiments had showed that these conditions "*completely destroyed"* concentrations of PSTVd similar to those found in PLRV virions isolated from doubly infected tissue. Cq-values of TCDVd-containing samples ranged between Cq=32 and Cq=39 after MNase treatment and were considered positive if the Cq-value was lower than 35. After cDNA synthesis of the samples, RT-PCR and capillary electrophoresis, clear amplicons were observed for MNase (0.001 U/µl) digested samples indicating that the viroid had not been fully degraded under these conditions.

Despite the different pospiviroid used in our tests (TCDVd) compared to the study of Querci *et al*. (1997, i.e. PSTVd) differences in MNase-degradation of TCDVd versus PSTVd were not expected. MNase is a highly unspecific enzyme that degrades all types of nucleic acids (double/single-stranded, circular/linear, /RNA/DNA) strands to di-en trinucleotides and both pospiviroids share a very high sequence homology (85-89%, Singh *et al.* 1999) and a highly similar secondary structure.

Furthermore, in two types of transmission experiments with aphids and doubly inoculated source plants, simultaneous transmission of virus and viroid (TASVd and TCDVd) did not take place. This observation is in contrast with previous tests, where PSTVd was used (Querci *et al.* 1997, Salazar *et al.* 1995, Singh & Kurz 1997, Syller *et al.* 1997, Syller & Marczewski 2001). The studies of Querci *et al.* (1997), Salazar *et al.* (1995), Singh & Kurz (1997), Syller *et al.* (1997) and Syller & Marczewski (2001) vary in numerous features, such as PSTVd isolate that was used for inoculation and the number and identity of source and receiving plants (i.e. *Datura stramonium, Physalis floridana*, *Nicotiana glutinosa*, *S. lycopersicum*, *S. tuberosum)*. Consequently, transmission efficiencies are also very different: 100% (Salazar *et al.* 1995), 20-60% (Querci *et al.* 1997), 3-14% (Syller *et al.* 1997), 0-55 % (Syller & Marczewski 2001), and 7% (Singh & Kurz 1997). The most obvious reason why TASVd and TCDVd were not transmitted in conjunction with PLRV as opposed to prevous studies, is the identity of the pospiviroid species used.

Indeed, as has been shown before for other viruses, encapsidation is a highly specific and regulated event, determined by specific recognition between the RNA or nucleocapsid complex and the coat proteins of the virus (Jia *et al.* 1998). It is therefore possible that it occurs for certain viroid species (or specific isolates) and not for others. Other reasons that may explain the differing results are: varying experimental conditions (e.g. length of the inoculation access period, plant cultivars used, temperature etc.) and a low prevalence of virus-assisted transmission of viroids in the form of "transencapsidation". It is recommended that future transencapsidation experiments with viroid species include PSTVd as a control, because this is the only pospiviroid for which transencapsidation has been shown before (Querci *et al.* 1997, Salazar *et al.* 1995, Singh & Kurz 1997, Syller *et al.* 1997, Syller & Marczewski 2001).

In conclusion, the MNase tests in this study did not result in a complete degradation of the TCDVd when applying the enzyme conditions of Querci *et al.* (1997). Additionally, PLRV-assisted transmission of TASVd and TCDVd to potato plants did not occur. However, it should be noted that more experiments (and replications) are needed in order to validate these preliminary results. Future studies should therefore try to unravel the potential associations between different viroid species/isolates and different (luteo)viruses further in order to be able to estimate epidemiological risks.

Chapter 7:

General discussion

7.1 RESEARCH FINDINGS AND FUTURE PERSPECTIVES

The general goal of this work was to investigate different transmission pathways of pospiviroids to gain a better understanding of pospiviroid epidemiology. To achieve this, specific aspects of the pathogen-vector-host triangle (Figure 7.1) were investigated: Pospiviroid-Host, Pospiviroid-Insect and Pospiviroid-Luteovirus interactions.

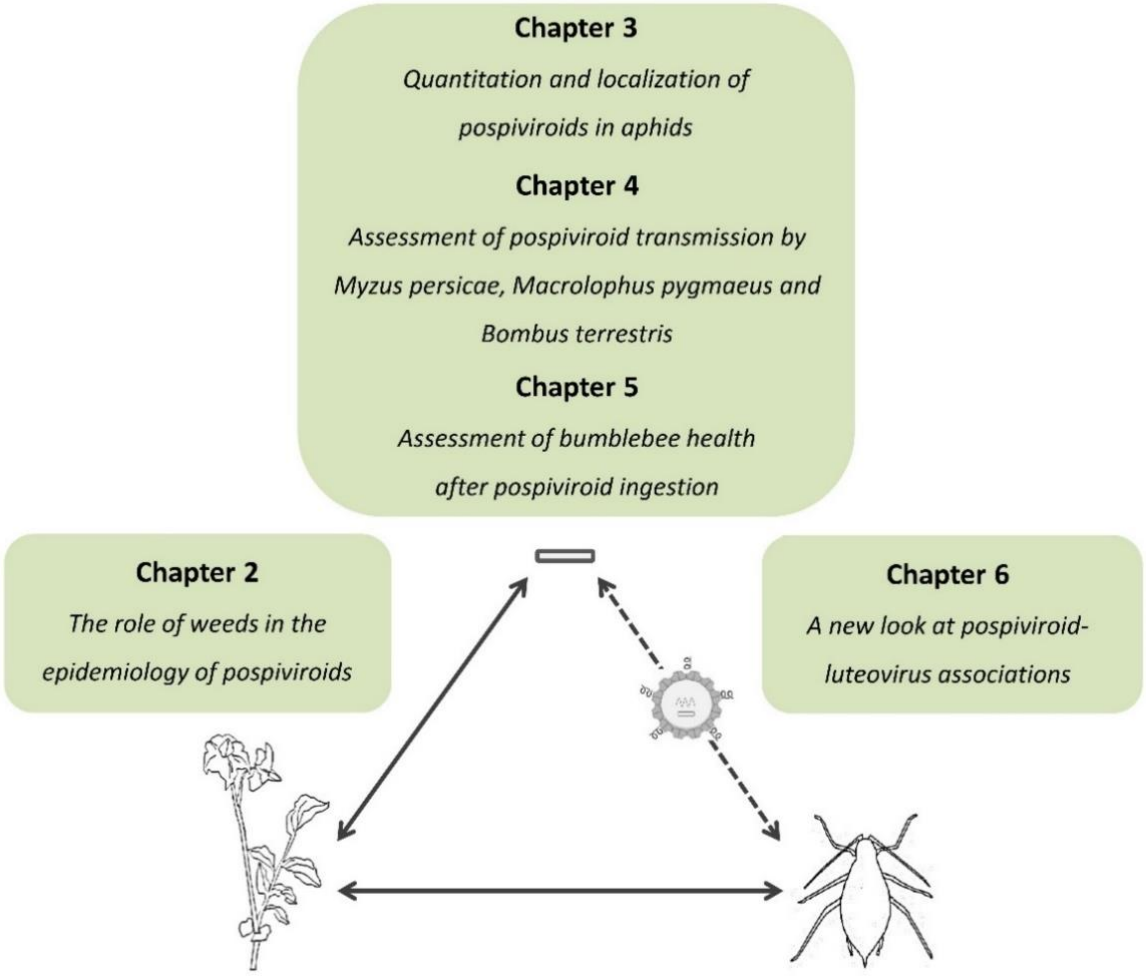


Figure 7.1 Schematic overview of the six research Chapters of this PhD.

- ***Pospiviroid-Host interactions***

In this PhD study we explored whether naturally occurring weeds could function as reservoirs for pospiviroids (Chapter 2). This question arose from the knowledge that many weeds are known as natural reservoirs of plant viruses (Cooper & Jones 2006, Duffus 1971). **Based on the results of our experiments, it was concluded that commonly occurring weed species in Belgian greenhouses do not appear to play a significant role as reservoir hosts for pospiviroids.**

This observation is confirmed by the results from recent inoculation studies with pospiviroids (Antignus *et al.* 2007, Matoušek *et al.* 2007), which showed that the natural host range of members of the Genus *Pospiviroid* is largely restricted to the family of the *Solanaceae* and to a lesser degree the family of the *Asteraceae*. Compared to the relatively narrow range of natural weed hosts for pospiviroids, many plant viruses, like *Cucumber mosaic virus* (CMV), have very broad host ranges, both experimentally and naturally (Palukaitis *et al.* 1992). It has been speculated that a high mutation frequency and a high degree of variation among isolates has allowed CMV to adapt to more niches (i.e. hosts) (Roossinck 1997).

It remains unclear why pospiviroid host plants are largely found in members belonging to the family of *Solanaceae*. Answering this question requires knowledge about the co-evolution of the pathogen and its host, its vector, the pathogen's replication mechanism, virulence determinants and plant-specific defense mechanisms (e.g. RNA silencing). Unlike viruses, viroids accomplish various functions of infection and spread by direct interaction of their RNA genome with the host factor(s) (Nie *et al.* 2005). Thus, isolation of viroids from various plant sources and determination of the secondary structure of viroid genomes constitutes the essential study of viroids and may provide an insight of the conserved RNA sequences involved in the adaptation of a viroid species in a particular host (Nie *et al.* 2005). In addition, inoculation assays and genomic analyses for host gene expression and characterization of replication intermediates could be helpful in investigating the susceptibility of plants for viroid infections in the future.

For future studies focusing on viroid host range, the choice of the inoculation technique is essential. Different results can be expected for the same viroid species depending on whether the inoculation was performed mechanically, or biolistically. Using a biolistic GeneGun, the viroid gets inserted in a precise location, directly into the plant cells, and therefore the infection process is aided substantially. Besides inoculation technique, differences in outcomes may also be caused by the (pospi)viroid species and/or the type of source plant (inoculum) that is being used for inoculation. For instance, during recent attempts to inoculate potato plants (cv. Kennebec) with TCDVd from *Vinca* sp., repeated mechanical inoculation trials were unsuccessful (Lisanne Devriese MSc Thesis, Ghent University, 2016). Therefore, an alternative inoculation procedure using a bridging host described in Verhoeven *et al.* (2016) was examined.

In the latter study, PSTVd RNA from an ornamental plant (*Dahlia*) was dissolved in inoculation buffer to infect tomato seedlings (in Verhoeven *et al.* 2016). Once the tomato got infected, a tomato leaf sample was used for the mechanical infection of potato (Verhoeven *et al.* 2016). When this procedure using tomato as a bridging host was used in our own experiments, potatoes got successfully infected with the TCDVd-isolate. This illustrates that tomato is susceptible to different pospiviroids from a variety of inoculum sources: an observation that is also reflected in phylogenetic trees of the genus *Pospiviroid* (Shiraishi *et al.* 2013, Verhoeven *et al*. 2004, Verhoeven *et al.* 2012). In these trees, sequences found in tomato are very diverse and dispersed over several clusters of the phylogenetic trees (Shiraishi *et al.* 2013, Verhoeven *et al*. 2004, Verhoeven *et al.* 2012).

There are many other potential weed hosts for pospiviroids that have not been tested in our study. It is likely that new pospiviroid hosts, as well as new viroid species and variants, will be identified in the future by the increased use of the Next-Generation Sequencing (NGS) technology. NGS is a rapid and high throughput sequencing method for RNA and DNA sequencing, gaining increasing popularity among viroid researchers (Barba *et al.* 2014, Boonham *et al.* 2014, Chiumenti *et al. 2*014, Fox *et al.* 2015, Li *et al.* 2012). NGS studies on viroids proved that this technique can be powerful for detecting viroids occurring in extremely low concentrations, for illuminating latent and mixed infections of viroids, discovering new viroid species and unravelling population dynamics of different viroid strains in plants (Barba *et al.* 2014, Boonham *et al.* 2014, Chiumenti *et al. 2*014, Fox *et al.* 2015, Li *et al.* 2012). Despite these interesting features of NGS, there are also some drawbacks, e.g. the variety of different pipelines and bio-informatic software decisions leading to divergent interpretations and results. To cope with these problems, interlaboratory validations will have to be organized in order to align NGS-procedures.

- ***Pospiviroid-Insect interactions***

Chapters 3 to 5 focus on the interactions between pospiviroids (= pathogen) and insects (= potential vectors). **Our results show that TASVd and PSTVd can be ingested by *M. persicae* while feeding on infected plants and can be reliably detected and localized using a combination of RT-qPCR and FISH in combination with confocal microscopy.**

The FISH-procedure used to localize viroids in aphids provides reliable, background-free results combined with much less time, effort and cost compared to other FISH-protocols with long processing times and costly materials (Ghanim *et al.* 2009). For future studies, a more precise ultrastructural localization could be achieved by conjugating the probes to a biotin-gold particle-streptavidin system and visualization through Transmission Electron Microscopy (TEM), as has been done for plant matrices by Bonfiglioli *et al.* (1996). To achieve a higher sensitivity and specificity, and to allow for multiplex detection of different viroids, the recently developed RNAscope® Technology could be tested (Wang *et al.* 2012, Figure 7.2). This technique differs from traditional FISH in that it is designed to amplify target-specific signals without amplifying the background, resulting in an improved signal-to-noise ratio (Wang *et al.* 2012, Figure 7.2).

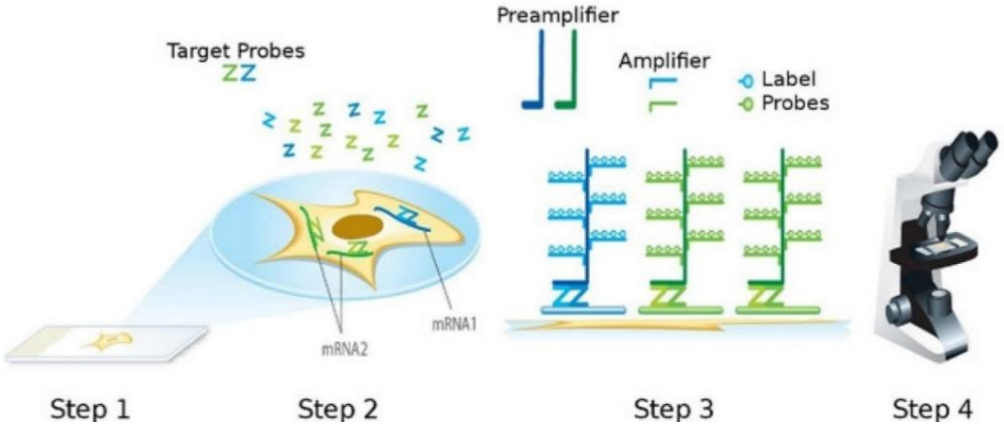


Figure 7.2: Schematic of the RNAscope assay procedure. In Step 1, tissues are fixed and permeabilized to allow for probe access. In Step 2, target RNA-specific oligonucleotide probes (Z) are hybridized in pairs (ZZ) to multiple RNA targets ("mRNA1/2"). In Step 3, multiple signal amplification molecules are hybridized, each recognizing a specific target probe, and each unique label probe is conjugated to a different fluorophore or enzyme. In Step 4, signals are detected using an epifluorescent microscope (for fluorescent label) or standard bright-field microscope (for enzyme label) (Wang *et al.* 2012).

In following transmission tests with *M. persicae*, *B. terrestris* and *M. pygmaeus*, both intra-and interspecies transmission of a variety of different host plants and four different pospiviroid species were tested. **We concluded that the presence of *M. persicae*, *B. terrestris* and *M. pygmaeus* in greenhouses does not imply a major phytosanitary risk for viroid dispersal.** However, it should be noted that generalizations to family level instead of species level can only be made for the family of the *Miridae* (Heteroptera), to which *M. pygmaeus* belongs.

The 20 mirid bugs that were tested after the acquisition period did not test positive for viroids and this insect was not able to transmit viroids. In a previous study, the mirid bug *Lygus lineolaris* was not able to transmit viroids either (Schumann *et al*. 1980). This may be explained by the fact that the stylets of these bugs cause too much damage to the plant cells whilst feeding (Mitchell 2004). For infection to occur a cell must both receive virus and remain functional and undamaged in the process (Nault 1997, Mitchell 2004). Generalising the aphid (Hemiptera: *Aphidoidea*) transmission results is more dangerous. In a study by De Bokx & Piron (1981) the aphid *M. euphorbiae* was able to transmit PSTVd, while this could not be achieved by other aphid species like *M. persicae* and *A. solani.* Furthermore, it is known that many aphid species have a specific relationship with certain plant viruses and that transmission efficiencies vary with aphid species (Ng & Perry 2004).

For the family of the *Apidae* (Hymenoptera), to which the honeybees and bumblebees belong, results cannot be generalized either. In our study, bumblebees were able to transmit TCDVd from infected petunias to tomato with a very low efficiency of only 2.6%. In a previous study, where intraspecific transmission of TCDVd by bumblebees was shown for tomato, 50% of the bumblebees transmitted the disease (Matsuura *et al.* 2010). In a study by Nielsen *et al.* (2012) bumblebees and honeybees did not transmit PSTVd. The differences in transmission results of bumblebees and honeybees may be explained by differences in experimental design (e.g. temperature or viroid species/isolate employed) or by differences in feeding behavior. In contrast to honeybees, bumblebees perform “buzz-polination” during which they grab the anthers of the flower and vibrate their bodies vigourously (Velthuis & Doorn 2006).

It is likely that the low transmission rate for bumblebees is caused by pollen-mediated transmission since most plant viruses that have floral visitor vectors are transmitted in this way (Card *et al.* 2007). These viruses are located in/on pollen grains, occasionally cause the pollen to become inviable and typically lead to systemic plant infections (McArt *et al.* 2014). In many cases, the infected pollen attaches mechanically to the exoskeleton of the insect during foraging on flowers and the disease is further vectored to plants when the pollen-associated virus detaches and enters (feeding) wounds in the plant’s tissues (McArt *et al.* 2014). To investigate whether ingestion of pospiviroid-infected pollen was passed on to bumblebee progeny, experiments were organized in which bumblebee microcolonies were fed with TASVd throughout a period of 50 days (Chapter 5).

We concluded that TASVd does not persist in bumblebee bodies and progeny after consumption of TASVd-infected pollen and that there are no negative effects on bumblebee colony formation.

For future work, it would be interesting to investigate which parameters influence insects in their decision to forage onto pospiviroid-infected or non-infected plants. It is known that plants use a variety of sensory signals (e.g. visual, olfactory, gustatory cues) to communicate with insects (Raguso & Willis 2002). In the case of latent infections caused by pospiviroids visual cues that could indicate an infection (i.e. disease symptoms) are absent. However, it is possible that plant-specific volatile components (olfactory/gustatory cues) influence insects in attraction or repulsion to latently infected or healthy plants. Plants synthesize and emit a large variety of volatile organic compounds with terpenoids, phynylpropanoids/benzenoids , fatty-acid and amino acid derivatives being the dominant classes (Dudareva *et al.* 2006). The primary functions of airborne volatiles are to defend plants against herbivores and pathogens or to provide a reproductive advantage by attracting pollinators and seed dispersers (Pichersky & Gershenzon 2002). It is currently unknown whether the volatile spectrum of viroid-infected plants influences attraction or repulsion of any kind of insect species. To answer this question, solid phase micro-extraction fibers (SPME) technology, in combination with gas-chromatography mass spectrometry (GC-MS) could be used to analyze the volatile fraction of viroid-infected flowers.

- ***Pospiviroid-Luteovirus interactions***

After finishing the insect transmission tests, the question still remains whether co-inoculation of the host plant with a pospiviroid and a luteovirus could lead to successful transmission. In Chapter 6 it was our goal to investigate transencapsidation for two other pospiviroids than PSTVd, namely TASVd and TCDVd, which share similar epidemiological (host range, symptoms) and sequence characteristics (partial sequence homology, similar secondary structure) with PSTVd. **Our results showed that during transmission tests with *M. persicae* and doubly infected *N. benthamiana* and *S. tuberosum*, the pospiviroids TCDVd and TASVd were not transmitted together with PLRV to new hosts.**

Additionally, after applying identical enzyme conditions as Querci *et al*. (1997) in combination with the more sensitive qPCR detection technique, a remnant quantity of TCDVd was detected. Since the enzyme degradation step has been crucial in the methodologies leading to (indirect) evidence for the phenomenon of transencapsidation (Querci *et al*. 1997, Syller *et al*. 1997) care should be taken in future experiments with nucleases.

A remnant concentration of viroid after MNase degradation could mistakenly be considered as "transencapsidated", i.e. protected from degradation because of its presence in a virus particle, while it is simply not being completely digested under the applied conditions. Future experiments should therefore test different enzyme concentrations as well as different viroid species/isolates in repeated assays in order to validate the working conditions of the enzyme. Even then, results must be interpreted with care, since *in vitro* conditions may result in different outcomes compared to what takes place *in vivo*. Besides testing different viroids, also other (luteo)viruses besides PLRV could be considered; e.g. the poleroviruses *Carrot red leaf virus* (CtRLV) and *Tobacco vein distorting virus* (TVDV), which are both known to encapsidate and transmit other viral RNAs (Huang *et al.* 2005, Mo *et al.* 2011). However, the selected virus and viroid should have an overlapping host range in the field, otherwise the epidemiological and economical relevance are lacking.

The papers of Querci *et al.* (1996) and Salazar *et al.* (1995) state that the occasional PSTVd contamination of PLRV isolates maintained at the International Potato Center (CIP, Lima, Peru) provided the first indication that PLRV might facilitate aphid transmission of PSTVd. The resulting studies on transencapsidation were an attempt to identify a possible reason of this co-occurrence in nature. Reports for natural mixed infections with other pospiviroid species than PSTVd, such as TCDVd and/or TASVd, are lacking. In Belgium, the occurrence of PSTVd and PLRV and PSTVd in potatoes is estimated very low. The incidence of PLRV has decreased drastically over the past years, thanks to an intensive monitoring and eradication procedure (personal communication, DCP ILVO). Furthermore, there are almost no reports of serious PSTVd-outbreaks in seed potatoes in EU-countries and consequently the risk for PSTVd-enabled transmission via PLRV and aphids is suspected to be extremely low.

Firm conclusions about the exact nature of the viroid-virus interaction cannot not be drawn at this instant. Therefore, as a baseline for future research, Figure 7.3 provides an overview of the different theoretical interactions that can be envisaged for pospiviroids and luteoviruses in a doubly infected plant. Luteoviruses, like PLRV, replicate in phloem companion and parenchyma cells (Mayo & Ziegler-Graff, 1996) and viroids follow the flow of photo-assimilates from the photosynthetic source to sink tissues through the phloem cells of the plant (Flores *et al.* 2005).

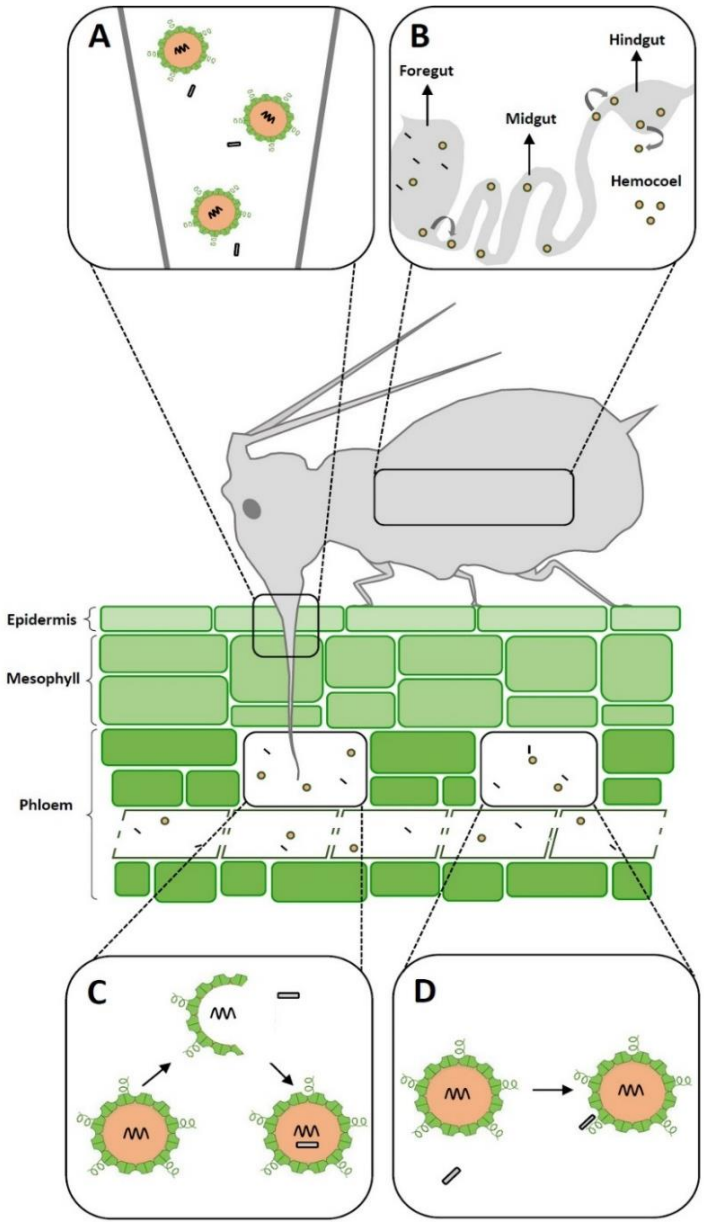


Figure 7.3: Aphid feeding on phloem cells in which viroids and luteoviruses are present. A-D represent four interactions scenarios: A = simultaneous, but unrelated, presence of viroids and luteoviruses in stylet, B = circulative, non-propagative transmission of luteoviruses in the aphid's body, C = virus assembly and transencapsidation, and D = viroid attachment to capsid proteins.

Therefore, when these pathogens are both present in phloem cells, viroids and viruses may interact: viroids could get encapsidated in virions (Figure 7.3 C) or they could attach to the viral capsid, e.g. to readthrough domains (RTDs, Figure 7.4), which are thought to regulate plant-virus-vector interactions (Chavez *et al.* 2012) (Figure 7.3 D).

Consequently, virions and viroids, or the association of both, could be taken up by an aphid, when its stylet pierces through a phloem cell (Figure 7.3 A). Inside the stylet and in the foregut it is possible that viroids are present, as was shown by FISH and confocal microscopy (Chapter 3). The icosahedral luteoviruses, with or without the viroid, will move through the insect's body in a circulative, non-propagative way (Figure 7.3 B). A combination of Panel A and B (Figure 7.3) is also a possibility: a circulative virus moving through the different body parts while the viroid remains in the mouthparts of the aphid, similarly as what happens to various non-persistent viruses.

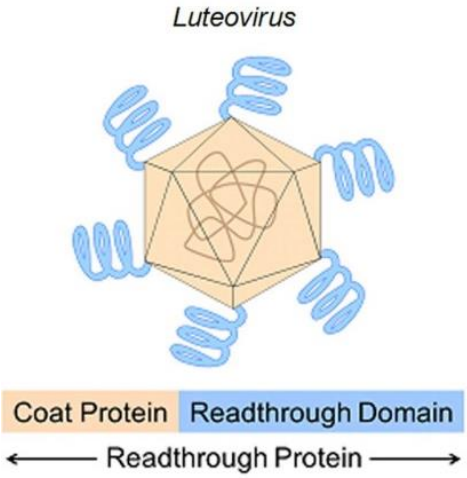


Figure 7.4: Structure of an icosahedral luteovirid showing the coat protein (CP) in pink and the read through domain (RTD) in blue (De Blasio *et al*. 2015).

Which of these different interaction scenario's will effectively take place will have to be studied in more detail in the future. Since direct viroid-protein interactions are known to occur (Daròs and Flores 2002, de Alba *et al.* 2003), it would be interesting to further investigate the interaction of pospiviroids with readthrough domains (RTDs) of the viral capsid. The group of Prof. Michelle Cilia (Cornell University) has explored several interesting tools that could helpful in investigating this interaction in the future.

For example, using a combination of co-immunoprecipitation, high resolution mass spectrometry and bio-informatics, host-PLRV-protein interactions were identified (DeBlasio *et al.* 2015). These techniques showed that the RTD expands the functional repertoire of the virus by interacting with a distinct set of host proteins (DeBlasio *et al.* 2015).

An alternative way of investigating these associations could be done through Cryo-Electron Microscopy (Cryo-EM) or X-ray crystallography. Latter technique allows visualization of the virus protein structure in great detail, but has the drawback of not being able to study the conformation of the nucleic acid within the virus particle. Cryo-EM involves rapid freezing of the materials, allowing specimens to be visualized in an undistorted way. Johnson *et al.* (2004) used "Virus Like Particles (VLPs)" of a nodavirus *Pariacoto virus* (PaV) to visualize the 3D-arrangement of the transencapsidated RNA by Cryo-EM image reconstruction. This study showed that factors other than the specific nucleotide sequence and other attributes of the genomic RNA are the main determinants of the formation of the structure (Johnson *et al.* 2004). Cryo-EM and X-ray crystallography could therefore be used to investigate the potential encapsidation of a viroid sequence in a viral particle (its precise location, folding, etc.) or the possibility that a viroid attaches to one of the outer capsid proteins of the virus.

In conclusion, this PhD thesis investigated interactions between pospiviroids (i.e. TASVd, TCDVd, PSTVd and PCFVd), insects (i.e. *M. persicae*, *B. terrestris*, *M. pygmaeus*), a luteovirus (PLRV) and various weed species in an experimental greenhouse context. Apart from the successful transmission of TCDVd by bumblebees, none of the studied pathways resulted in significant transmission events. In order to broaden the discussion, and to estimate the risks associated to pospiviroid species in Belgium, different parameters that influence pospiviroid incidence and transmission are discussed in more detail in the following part (7.2 Risks for Belgium).

7.2 RISKS FOR BELGIUM

7.2.1 Pest Risk Assessments for pospiviroids

According to the "International Plant Protection Convention" (IPPC) and the "World Trade Organization Agreement on the Application of Sanitary and Phytosanitary measures" (WTOSPS Agreement, WTO, 2009), any measure aimed at preventing the introduction and spread of new pests must be justified by a science-based Pest Risk Analysis (PRA). PRA is a process that evaluates technical, scientific and economic evidence to determine whether an organism shall be categorized as quarantine pest and, if so, how it should be managed (FAO 2016). Since 2006, supra-national bodies such as EPPO and the European Food Safety Authority (EFSA) started to conduct PRA at regional or EU level. A PRA of solanaceous pospiviroids for the EU territory was published in 2011 following a request of the EC (EFSA Panel on Plant Health, 2011). In this PRA, various entry pathways, mainly involving plant propagation material, were identified (EFSA Panel on Plant Health, 2011). The PRA states that uncertainties mainly derive from a limited knowledge on pospiviroids other than PSTVd, although all pospiviroids are expected to have similar biological properties (EFSA Panel on Plant Health, 2011). On insect transmission, the PRA states that transmission of pospiviroids by aphids or bumblebees, within and between crops, has an "unlikely to moderately likely" probability rating (EFSA Panel on Plant Health, 2011). Additionally, the PRA states that "high uncertainties on this assessment derive from the limited number of virus-viroid-host-vector combinations for which experimental data are available."

While many European research projects, as well as the PRA conducted in 2011, have elicited detailed information on the epidemiology of pospiviroids, only one study has performed a quantitative economic risk assessment. To assess the economic impact of PSTVd in Europe, Soliman *et al.* (2012a-b) developed an analytical model for potato and tomato. Using stochastic simulations, the total economic impact in Europe was estimated at 4.4 million euros for potatoes and 5.7 million euros for tomatoes (Soliman *et al.* 2012a-b). This analytic model consisted out of four main components, 1) the infestation level which is the proportion of potato and tomato plants infected with PSTVd), 2) a climate component to describe the climate suitability for damage expression, 3) a host component to determine the spatial distribution and value of hosts, and 4) and an economic component (Soliman *et al.* 2012a-b).

Combining the information obtained throughout this PhD-study with information gathered through national projects and European collaborations, we evaluated these four risk assessment components for food crops and ornamental plants in Belgium. Additionally, two other components were added: i.e. pathogens and vectors (Table 7.1). Each of the components is discussed below and a color score that represents the risk is provided in Table 7.1.

Table 7.1 An overview of 6 risk assessment components for pospiviroids in Belgium. Color score = estimated risk (light grey = low, medium grey = intermediate, dark grey = high).

PRA Component	**Food crops**			**Ornamental plants**	
	Potato	Tomato	Pepper	Chrysanthemum	Other
Pathogen					
Vector					
Host					
Infestation level					
Climatic conditions					
Economic impact					

For the **Pathogen** component (Table 7.1), pospiviroid phylogenies with Belgian isolates suggest that contaminations from infected ornamentals to certain susceptible vegetable crops (e.g. tomato, pepper) have occurred in the past. For potato, no natural infections have occurred in Belgium. CSVd-infections in chrysanths have been found in the past, but eradication and increased awareness among the growers has led to a decrease in the number of infections. In contrast, many viroid species are still found in other ornamental plants all over Belgium, many of which originate from abroad. Risks for the **Vector** component are estimated low to intermediate, based on the following reasoning:

1) No evidence can be provided for viroid transencapsidation and transmission through PLRV-vectors in outdoor potatoes in Europe;
2) In glasshouse crops that are pollinated using commercial bumblebee hives (e.g. tomato, pepper) our research pointed to a very low chance of transmission;
3) Direct transmission by pest insects (like aphids) has not been observed during this thesis. Additionally, pest control in commercial glasshouses is quite severe since plants have to be in a unspoiled state in order to be sold. Therefore, insects are often immediately eradicated if found.

It is clear that Belgium offers a wide range of suitable hosts for pospiviroids (**Host** component, Table 7.1). Various pospiviroid hosts are present in the group of the (solanaceous) outdoor and glasshouse crops (potato, tomato, eggplants, peppers) and among the solanaceous ornamental plants. Luckily, no new pospiviroid hosts were found among commonly occurring weed plants in Belgium during this PhD-research. One of the most difficult components to estimate, is the **Infestation level** of pospiviroids in Belgium (Table 7.1). Experts estimated the PSTVd infestation level in Europe to vary between a range of 0 to 10% (Soliman *et al.* 2012a). Apart from two outbreaks in tomatoes in 1996 and 2006 (Verhoeven *et al*. 2004, Verhoeven *et al.* 2007) there are no other recordings of pospiviroid infections in Belgian vegetable crops. Based on our own findings and other reports, the infestation level of pospiviroids in ornamental plants is higher than the infestation level in food crops (Table 7.1).

As for the **Climatic conditions** (Table 7.1), it has been demonstrated before that higher air temperatures will promote viroid replication in plants: in potato tissues, viroid concentration doubled as temperatures rose from 25°C to 30°C (Morris & Smith 1977). Additionally, the survival temperature range for pospiviroids is quite large: for instance, it has been shown that TCDVd can survive at subzero temperatures (Singh 2014, Singh & Dilworth 2009, TOPOVIR 2011). Therefore, the Belgian climate characterized by moderate winters, does qualify as a region where pospiviroids could replicate and survive in food crops tissues. However, reports of viroid outbreaks in field crops are lacking. Many ornamental flowering plants are grown in warmer, subtropical climates. The Belgian chrysanthemum breeding industry has growing facilities in South-America and Africa. Solanaceous ornamentals that are imported into Belgium often originate from Mediterranean countries, like Italy, Portugal and Israel. Once in Belgium, these plants are usually maintained inside glasshouses. Production conditions and cultivation techniques of host plants are also important to consider: differences in viroid transmission can be expected depending on greenhouse vs. field conditions, manual vs. mechanized harvesting, the use of intercropping and hygienic conditions.

Lastly, for the **Economic impact** component, it is estimated that there is a low impact for outdoor potato production and indoor tomato and pepper cultivation (Table 7.1). In the top 10 of most producing potato and tomato countries of the EU, Belgium grows a significant portion of the worldwide production of these two crops.

During a field trial in Canada, yield reductions due to PSTVd-infections in potato were 17-24% for mild strains and up to 64% for severe strains (Singh *et al.* 1971). In general, estimations of yield effects caused by pospiviroids in commercial potato and tomato productions are poorly documented. No significant economic impact is expected for ornamental plants (besides chrysanthemums) since most infections are latent. In this sector, economic losses manifest themselves in the eradication of plants after positive detection results of quarantine-regulated viroids. In conclusion, most of the combinations of PRA components and hosts correspond to "low" and "intermediate" risks (light/medium grey cells, Table 7.1). Table 7.1 shows "high risks" (dark grey cells for Pathogen, Host and Infestation level) in the case of ornamental plants (other than chrysanthemum) since these plants are abundantly present in Belgium and pospiviroid incidence in these plants is estimated to be high. Therefore, vigilance is still required, especially in the case of tomato, which can be easily infected with a variety of pospiviroid infection sources.

7.2.2 Future legislation of pospiviroids in the EU

Meanwhile, it has been shown that pospiviroids, other than PSTVd, can be at least as harmful to potato and tomato as PSTVd (Verhoeven *et al.* 2004). In tomato, similar symptoms and transmission modes were observed irrespective of the viroid concerned (Verhoeven 2010e). Additionally, many pospiviroid species are ubiquitously present in solanaceous ornamental hosts (Singh 2006a; Verhoeven *et al.* 2008a). Under the current legislation, ornamental plants that are found infected with PSTVd in Belgium need to be reported to the FASFC and consequently, plants will be destroyed. This measure is drastic, since disease symptoms in these plants are lacking and the risk of transmission to other vulnerable crops is small. It may be expected that in the future the European regulation concerning pospiviroids will be reformed so that regulation measures for pospiviroids will only hold for specific plant species and matrices, but not for all (personal communication dr. Verhoeven). For instance, it seems logical to lose the q-status of PSTVd (and other pospiviroids) in ornamental plants, but maintain it for specific crops, e.g. potato. For seed crops like tomato, pepper and eggplants a q-status for pospiviroids could be limited for the matrix "seed" only. By using specific measures like these future outbreaks can be minimized whilst maintaining a profitable and durable plant production industry.

7.2.3 Phytosanitary measures: prevention and control

To prevent the introduction of pospiviroid-infected plant material into the field or into the greenhouse, phytosanitary certification of planting materials is extremely important (Kovalskaya & Hammond 2014, Singh *et al.* 2003). In addition to phytosanitary certification, there are a number of other biosecurity measures that can be taken to avoid viroid outbreaks (Table 7.2). The most important control measures for viroids are: reducing transmission by strict hygienic procedures, the use of viroid-free seed and planting material and a continuous monitoring of plants followed by eradication (when plants are infected) and follow-up after the viroid outbreak (Table 7.2).

This PhD-study has showed that transmission by *M. persicae* and *M. pygmaeus* failed to transmit the examined pospiviroids, whereas for *B. terrestris* only a limited transmission was observed for TCDVd. Additionally, no naturally occurring weed hosts were identified amongst the tested species. Therefore, one of the most important transmission routes for viroids remains mechanical transmission: i.e. inoculation due to close contact with infected plants, plant handling, contaminated tools. Verhoeven *et al.* (2010c) showed that for mechanical transmission, temperature, plant species and source of inoculum are critical factors. An average temperature of 15°C only results in a few infections, whereas transmission at 25°C is more successful (Verhoeven *et al.* 2010). Since pospiviroid-affected plants are often cultivated in greenhouses or are subjected to a lot of handling, effective disinfection measures to clean cultivating tools, machineries and facilities are vital (EFSA Panel on Plant Health, 2011). Olivier *et al.* (2015) observed that effective disinfection can be achieved using Virocid™; Hyprelva™ SL; Virkon® and Jet 5® and bleach, but not with MENNO® clean, an approved product for PSTVd in several European countries. In a study by Mackie *et al.* (2015) the most successful disinfectans to inactivate PSTVd infectivity in infective sap were 20% nonfat dried skim mild and a 1:4 dilution of household bleach (active ingredient sodium hypochlorite). Additionally, this study showed that PSTVd remained infectious for 24h on various kinds of surfaces, like leather, plastic or string (Mackie *et al.* 2015).

Table 7.2 An overview of phytosanitary measures for pospiviroids, including practical examples. References in the third column are studies that have focused on each of the phytosanitary measures and offer more information on each topic.

Phytosanitary measure	Examples	References
Phytosanitary certification	In Europe PSTVd is classified as both an EU Annex 1/A1 and an EPPO A2 quarantine organism. This means that seed potatoes must be certified as free of PSTVd before export. In many other countries similar certification schemes are required. Hence, PSTVd has virtually been eliminated from potato production areas in the EU because it is no longer introduced into the field each growing season via contaminated seed potato.	Morris & Smith (1977) Singh & Crowley (1985) Salazar & Kahn (1989) EU emergency measures (2007/410/EC) De Hoop *et al.* (2008) Rodoni (2009)
Reducing transmission	Avoiding mechanical contamination (e.g. by physically separating cultivations like ornamental plants and crops) and strict hygienic conditions (e.g. the decontamination of equipment).	EFSA (2011) Olivier *et al.* (2015)
Monitoring, eradication and follow-up of outbreaks	Monitoring crops for unusual symptoms can lead to an early diagnosis and eradication (if necessary). For potato, EPPO developed a national regulatory control system for PSTVd that provides guidance on preventing its introduction, surveillance for the pathogen and its containment and eradication if found infecting potato plants or tubers.	Morris & Smith (1977) Singh & Crowley (1985) Hailstones *et al.* (2003) Sun *et al.* (2004) De Boer & DeHaan (2005)

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Summary

Since the discovery of the *Potato spindle tuber viroid* (PSTVd, Diener 1971) researchers all over the globe have investigated the unique molecular and epidemiological features of pospiviroids. Trending topics in viroid research during the past decades were: viroid replication, molecular and biochemical features of viroids, RNA-silencing, host range and transmission mechanisms. Less investigated topics were those related to the interaction with insects and weeds and the potential transencapsidation by plant viruses. This PhD therefore aimed to elucidate several aspects of the pathogen-vector-host triangle.

Survey results, together with contact-and inoculation experiments using weed plants and pospiviroids in the period 2012-2014, showed that commonly occurring weeds do not appear to play a significant role as reservoir hosts for pospiviroids. Only 5% of the inoculated weeds, tested in the current and in previous studies, can serve as an experimental host for viroids. The natural host range of viroids therefore remains mostly constricted to the families of the *Solanaceae* and the *Asteraceae*.

Fluorescence *in situ* hybridization experiments with aphids (*M. persicae*) that had fed on pospiviroid-infected plants, revealed fluorescent TASVd signals in the aphid's stylet and digestive system, but not in the embryos. In subsequent transmission experiments, *M. persicae* did not succeed in transmitting the TASVd to healthy host plants. Also insects from other functional groups were tested as potential vectors: i.e. *B. terrestris* (pollinators) and *M. pygmaeus* (biological control agents). The results from these experiments indicate that, although individual insects often test positive after the acquisition period, plants do not get easily infected after insect foraging.

A small-scale experiment was organized to assess whether the plant-pathogenic TASVd could infect bumblebees and evoke negative results on colony formation by delivering TASVd-infected pollen to ten microcolonies (in total) over a period of 50 days. After testing with RT-qPCR none of the tested lifestages (eggs, larvae, pupae, worker bees) tested positive for TASVd, indicating that this viroid does not to replicate in bumblebees. Additionally, in these colonies it seemed that colony formation developed normally, with a similar timing of the appearance of each of the lifestages and a similar total biomass after 50 days.

In previous studies focusing on transencapsidation of a viroid into a virus, the activity of the enzyme Micrococcal Nuclease (MNase) was crucial in providing evidence for this phenomenon. Our results showed that MNase (at a concentration of 0.001 U/µl) does not degrade the pospiviroid TCDVd completely. To investigate whether insects could transmit other viroids than PSTVd the presence of an assisting virus, experiments were organized with TASVd/TCDVd and PLRV-infected hosts and *M. persicae* as a vector. However, virus-assisted transmission of these two pospiviroids between plants, did not occur in any of our experiments, and neither has it ever been reported to occur in European field potatoes.

In Belgium there is a general decreasing trend in quarantine pospiviroids due to routine monitoring and eradication, in combination with a general increased awareness, but still pospiviroids are ubiquitously present in many ornamentals. Therefore, this pool of symptomless-infected ornamental plants can still pose a risk to economically important plants in Belgium like tomatoes, peppers and chrysanthemums.

Samenvatting

Sinds de ontdekking van het aardappelspindelknolviroïde (PSTVd, Diener 1971) hebben tal van studies unieke moleculaire en epidemiologische eigenaardigheden van viroïden blootgelegd. Onderwerpen als replicatie, moleculaire en biochemische eigenschappen van viroïden, RNA-silencing, waardplantenbereik en transmissiemechanismen konden op veel wetenschappelijke aandacht rekenen. Minder onderzochte topics waren insectentransmissie, het belang van onkruiden en de potentiële transencapsidatie door plantenvirussen. Dit doctoraat had dan ook de doelstelling deze aspecten van de pathogeen-vector-gastheer driehoek verder te ontrafelen.

Resultaten afkomstig van een survey, een contact- en een inoculatie experiment met onkruiden in de periode 2012-2014 tonen aan dat de geteste, algemeen voorkomende onkruiden geen significante rol spelen als reservoirs voor pospiviroïden. Slechts 5% van alle geïnoculeerde onkruiden uit de huidige en voorgaande studies kan dienen als experimentele gastheer voor viroïden. Het natuurlijke waardplantenbereik van viroïden blijft dus voornamelijk beperkt tot de families van de *Solanaceae* en de *Asteraceae*.

Fluorescentie *in situ* hybridisatie experimenten met bladluizen (*M. persicae*) die zich gevoed hadden met TASVd-geïnfecteerde planten, leidde tot de ontdekking van fluorescente viroïdensignalen in het spijsverteringstelsel van de bladluis. Deze signalen ontbraken in de bladluizenembryos. In daaropvolgende transmissie-experimenten, slaagden *M. persicae* individuen er niet in om TASVd over te dragen naar gezonde waardplanten. Ook insecten van andere functionele groepen werden getest: i.e. *B. terrestris* (pollinatoren) en *M. pygmaeus* (biologische bestrijders). Afgezien van het feit dat individuele insecten positief kunnen testen na de acquisitieperiode, tonen de resultaten van deze experimenten aan dat waardplanten niet gemakkelijk geïnfecteerd geraken na een insectenbezoek. In experimenten waarin hommels gevoed werden met TASVd-besmet pollen, bleek dit viroïde niet in staat om hommels te infecteren en werden geen negatieve effecten waargenomen op de ontwikkeling van hommel microkolonies.

In voorgaande studies over transencapsidatie van viroïden in virussen, was de activiteit van het enzyme Micrococcal Nuclease (MNase) van cruciaal belang in de bewijsvoering voor dit fenomeen. Onze resultaten tonen echter aan dat MNase bij een concentratie van 0.001 U/µl niet al het aanwezige TCDVd afbreekt.

Om na te gaan of insecten viroïden kunnen overdragen in het bijzijn van een assisterend virus, werden experimenten georganiseerd met TCDVd/TASVd en PLRV-geïnfecteerde gastheren en *M. persicae* als vector. In deze experimenten werd virus-geassisteerde transmissie van deze twee pospiviroïden tussen planten niet waargenomen. Daarenboven is het fenomeen ook nog niet gerapporteerd geweest in Europees pootgoed.

In België is er een algemeen dalende trend in quarantaine pospiviroïden tengevolge van routine monitoring en uitroeiing in het geval van besmetting. Daarnaast is er onder telers een groeiend bewustzijn over het belang van ontsmetting en hygeïnische praktijken om viroïdenbesmettingen te voorkomen. Desondanks de getroffen maatregelen zijn pospiviroïden nog steeds wijdverspreid aanwezig in tal van sierplanten. Deze sierplanten vormen bijgevolg nog steeds een risico voor economisch belangrijke gewassen als tomaat, paprika en chrysant.

Curriculum Vitae

Personal data

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Education

2012 **MSc** "Master-after-master in Environmental Sanitation and Law" (Ghent University, Belgium) *with distinction*.

Thesis title: "Mutual interactions between viruses and the gut microbiota of bumblebees (*Bombus terrestris*, L.)". Promotor: Prof. dr. ir. Guy Smagghe (Ghent University), Co-promotor: Dr. Ivan Meeus (Ghent University).

2011 **MSc** "Erasmus Mundus in Marine Biodiversity and Conservation" (EMBC, Ghent University, Belgium) *with great distinction*.

Thesis title: "Modelling available data of turbot (*Scophthalmus maximus*) in the Irish and Celtic Seas: A first step towards sustainable management?". Promotor: Dr. Marijn Rabaut (Ghent University), Co-promotors: Ir. Els Torreele (ILVO), Dr. Jan Jaap Poos (IMARES, Wageningen Universiteit, the Netherlands).

2009 **BSc** in Biology (Ghent University, Belgium) *with distinction*.

2006 Secondary school degree, Latin-Mathematics, Koninklijk Atheneum Sint-Niklaas

Awards/grants

2016 B.A.E.F. Fellowship for Post-Doctoral Research in the US

Doctoral training courses

2012 Gene technology and Molecular Diagnostics (Ghent University, Belgium)

2013 Hands-on Light-Microscopy Workshop (University of Luxemburg, Luxemburg)

2014 Non-parametric statistics (Ghent University, Belgium)

Publications

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1. Van Bogaert, N., De Jonghe, K., Van Damme, E.J.M., Maes, M. and Smagghe, G. (2015). Quantitation and localization of pospiviroids in aphids. *Journal of Virological Methods*, 211, 51-54.
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Oral presentations

2013 International Advances in Plant Virology (IAPV, Norwich, UK)

2014 66th International Symposium on Crop Protection (ISCP, Ghent Unversity, BE)

2015 International Conference on Viroids and Viroid-like RNAs (Ceske Budejovice, CR)

2016 68th International Symposium on Crop Protection (ISCP, Ghent Unversity, BE)

Poster presentations

2012 Hands-on Light-Microscopy Workshop (University of Luxemburg, Luxemburg)

2014 19th Triennial Conference of the European Association for Potato Research (EAPR, Brussels, BE)

2015 67th International Symposium on Crop Protection (ISCP, Ghent Unversity, BE)

2015 International Conference on Viroids and Viroid-like RNAs (Ceske Budejovice, CR)

Summer courses

2014 "Fish Stock Assessment- Introduction" (ICES, Copenhagen, DE)

2010 "EMBC" summer school in Malta

2009 "DAM" (Diversity of Marine Algae, Roscoff biological station, FR)

2008 "BIOMA" (Biological quality of Marine waters, Arago Laboratoty, Banyuls-sur-mer, FR)

Supervision of graduate students

2012-2013 Niels Victoor (BSc. HoGent)

2013-2014 Lieselot Van Steendam (MSc. UGent)

2014-2015 Yoika Foucart (BSc. Odisee), Karel Monsieur (BSc. HoGent)

2015-2016 Lisanne Devriese (MSc. UGent)